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**Role of Antibodies and T cells in  
Pigeon Fanciers' Lung**

**Dr Zohreh Nademi**

**PhD Thesis**

**2008**

# **Role of Antibodies and T cells in Pigeon Fanciers' Lung**

**A thesis submitted**

**By**

**Dr Zohreh Nademi**

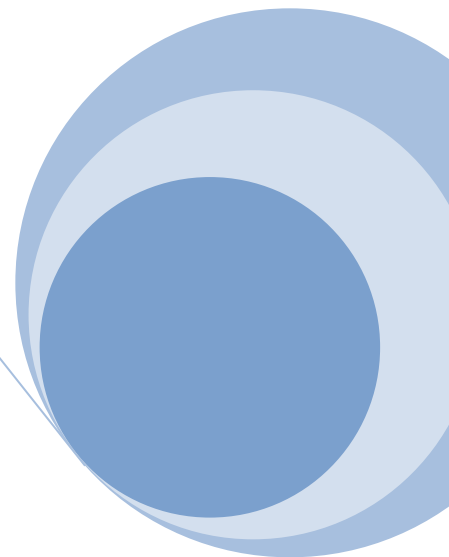
**MD**

**In partial fulfilment of  
the requirement for the degree of**

**DOCTOR OF PHILOSOPHY**

**School of Applied Science**

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## **Abstract:**

**Introduction:** Pigeon fanciers' lung (PFL) is one of the most common forms of hypersensitivity pneumonitis (HP) in the UK. Generally it is considered that PFL is caused by immune complexes, however, this does not explain why some fanciers are asymptomatic despite the presence of high levels of anti-avian antigen antibodies in their serum. Pigeon intestinal mucin (PIM) is considered to be an important antigen in PFL. Thus this study was designed in order to understand the role of specific antibodies and T cells in the pathogenesis of PFL.

**Methods:** Anti-avian IgG and IgG subclass responses among 50 symptomatic and 50 asymptomatic pigeon fanciers were determined by ELISA and the functional affinity of IgG1 and IgG2 against a range of pigeon antigens was determined by inhibition ELISA and microcalimetry. Mucin-specific T cell clones were also generated from pigeon fanciers and T cell phenotypes and cytokine profile of these cells were identified.

**Results:** The median titres of IgG1 and IgG2 against all the pigeon antigens tested was always higher in asymptomatic than symptomatic fanciers and these differences were significant for anti-PS IgG1 ( $P=0.04$ ), anti-PDF IgG2 ( $P=0.028$ ), anti-PDO IgG2 ( $P=0.04$ ) and anti-PIS IgG2 ( $P=0.03$ ). The functional affinity of IgG1 and IgG2 against PDO was higher in symptomatic individuals as compared to asymptomatic fanciers ( $P=0.006$  and  $P=0.002$ , respectively) whilst the functional affinity of anti-PDF IgG2 was also significantly higher in these patients ( $P\leq 0.001$ ). Symptomatic fanciers were also significantly more likely to have high  $\Delta H$  and thus had higher avidity antibodies against PDO ( $P=0.044$ ). 12 T cell clones specific for t mucin also were generated from an asymptomatic fancier and 90-96% of clone 04, 22, 23 were CD4<sup>+</sup>CD8<sup>-</sup> double negative (DN).

**Conclusion:** The data suggests that the magnitude of the serum antibody response cannot determine the development of the disease and as symptomatic fanciers had higher IgG antibody avidities and therefore immune complexes in individuals with PFL may have a stronger composition and bonds. In addition, this is the first demonstration of the use of ITC to measure antibody avidity in a clinical situation. This is a rapid and simple method of measuring antibody avidity and has a diagnostic potential in PFL. Finally t mucin-specific T cell clones with double negative phenotype may have a crucial role in immune regulation in asymptomatic fanciers and can be one of the reasons why these individuals do not have any symptoms in spite of having high antibody responses.

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**Declaration:**

This thesis is submitted by the undersigned to the School of Applied Science of University of Northumbria in Newcastle in partial fulfilment of the requirement for the degree of Doctor of Philosophy. The author confirms that this work is original and has not been submitted for any other award or institution, either in the UK or elsewhere.

Dr Zohreh Nademi

November 2008

## **1: Introduction**

### **1.1: Hypersensitivity Pneumonitis:**

#### **1.1.1: Definition:**

Hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis (EAA) is a group of inflammatory interstitial lung diseases resulting from hypersensitivity reactions after the repeated inhalation or ingestion of certain low molecular weight organic dusts or antigens derived from fungal, bacterial, animal protein or chemical sources (Fink *et al.*, 1968; Kokkarinen, Tukiainen & Terho, 1993; Schuyler *et al.*, 1997; Wahlstrom *et al.*, 1997; Kurup, Zacharisen & Fink, 2006; Madison, 2008). Provoking antigens associated with HP are usually less than 3 microns diameter which enable them to reach to the distal bronchial tree and alveoli in the lung where they may be transferred to the hilar nodes via the lymphatic system and therefore may induce an immune response including systemic IgG antibody production (Bourke *et al.*, 2003). In contrast those antigens provoking asthma are larger and tend to be deposited in the proximal airways, stimulating an IgE response in atopic individuals (Calvert *et al.*, 1999). However, even larger antigens may be able to reach the distal airways and alveoli after degradation and dissolving in lung secretions.

Less than 10% of the individuals exposed to provoking antigens may develop HP (Bourke *et al.*, 2003). In susceptible subjects hypersensitivity responses may result in a diffuse inflammation of the lung parenchyma and granulomatous inflammation of the distal bronchioles and alveoli (Fink, 1992; Kaltreider, 1993; Selman, 2004).

### **1.1.2: Epidemiology:**

Prevalence rates of HP are different throughout the world (Bourke *et al.*, 2001). Different factors including climate, geographical conditions, smoking habit, local customs and working practices have been suggested to influence the prevalence rates (Terho, Husman & Vohlonen, 1987; Bourke *et al.*, 2001; Takahashi *et al.*, 2002; Fink *et al.*, 2005; Solaymani-Dodaran *et al.*, 2007).

Overall the annual incidence of interstitial lung disease has been estimated to be 30:100000 in the general population with HP accounting for almost 2% of these cases (Coultas *et al.*, 1994; Bourke *et al.*, 2003). The incidence rate of HP was reported as approximately 1:100000 in the UK per year between 1991 and 2003, and therefore about 600 new cases of HP may occur each year in the UK (Solaymani-Dodaran *et al.*, 2007). Among different forms of HP, bird fanciers' lung was reported as one of the most common forms in the UK due to the popularity of keeping budgerigars (Bourke *et al.*, 2003) and the prevalence has been estimated to be 0.5-7.5% among bird owners (Hendrick, Faux & Marshall, 1978; Judson & Sahn, 2004). There are 80000 registered pigeon fanciers in the UK (McSharry *et al.*, 2006a) and 5-6 million homes in Britain harbour budgerigars. Thus Hendrick *et al.* suggested that budgerigar lung may be the most common type of HP in the UK (Hendrick, Faux & Marshall, 1978). On the other hand, the prevalence of pigeon fanciers' lung (PFL) was estimated to be 20-20000 cases per 100000 populations at risk in the US (Sharma, 2006).

Another form of HP is farmer's lung with an estimated prevalence of 10-200:100000 in population at risk in England (Staines & Forman, 1961) and more recently 420-3000 cases per 100000 populations at risk in the US (Sharma, 2006).

The prevalence of farmer's lung in exposed populations in some other European countries has been estimated at between 0.5- 3% (Lacasse & Cormier, 2006) depending on climatic

conditions and farming practice in harvesting and storage of hay and crops (Dalphin *et al.*, 1991).

### **1.1.3: Aetiology:**

Over the last 3-4 decades a number of different antigens have been identified as causative antigens and provocative materials for HP (Fink, 1992; Guillon *et al.*, 1992; Kaltreider, 1993; Apostolakos, Rossmoore & Beckett, 2001; Bourke *et al.*, 2003; Kurup, Zacharisen & Fink, 2006; Lacasse & Cormier, 2006).

There are large number of different forms of HP and causative antigens and a number of them are shown in Table 1.1.

Disease	Source of Exposure	Antigens
Animal handler lung	Urine, serum, fur	Animal proteins
Bagassosis	Mouldy bagasse (pressed sugarcane)	Bacteria ( <i>Thermophilic actinomyces saccharii</i> , <i>Thermoactinomyces vulgaris</i> )
Basement shower HP	Mouldy basement shower, mouldy home	<i>Epicoccum nigrum</i> , <i>Fusarium napiforme</i>
Bird breeder lung such as pigeon fanciers' lung (PFL)	Bird droppings and feathers	Avian glycoproteins
Cheese washer lung	Cheese casings	Fungus ( <i>Penicillium casei</i> or <i>P. roqueforti</i> )
Chemical worker's lung	Manufacture of plastics, polyurethane foam, rubber	Trimellitic anhydride, Diisocyanate, Methylene diisocyanate
Coffee worker's lung Tea grower's lung	Coffee bean dust, Tea leaves	Coffee and tea dust
Compost HP	Compost	Fungus ( <i>Aspergillus</i> )
Drug-induced HP	Medication	Amiodaron, Gold, Minocycline Hydrochloride, Clozapine, Nitrofurantoin, Fluoxetine, Beta blockers, Procarbazine, Cyclosporine
Dry rot HP	Mouldy rotten wood	Fungus ( <i>Merulius lacrymans</i> )
El Nino lung	Mouldy home from flooding	<i>Pezizia domiciliana</i>

**Table 1.1:** Some of the various types and the causative antigens of hypersensitivity pneumonitis (HP); Toluene diisocyanate (TDI), Hexamethylene diisocyanate (HDI), Methylenediphenyl diisocyanate (MDI) and Trimellitic anhydride (TMA) (Fink, 1992; Guillon *et al.*, 1992; Kaltreider, 1993; Apostolakos, Rossmore & Beckett, 2001; Bourke *et al.*, 2003; Kurup, Zacharisen & Fink, 2006; Lacasse & Cormier, 2006).



<b>Enzyme/detergent worker's lung</b>	<b>Enzyme dust, contaminated house dust</b>	<b>Bacteria (<i>Bacillus subtilis</i>)</b>
<b>Esparto grass HP</b>	Mouldy esparto used to produce ropes, canvas, sandals, mats, baskets, and paper paste;	Fungus ( <i>Aspergillus fumigatus</i> )
<b>Farmer's lung</b>	Mouldy hay	Bacteria ( <i>Thermophilic actinomycetes</i> , <i>Saccaropolyspora rectivirgula</i> )  Fungus ( <i>Aspergillus species</i> )
<b>Floor finisher's lung</b>	Mouldy wood floor	<i>Cephalosporium acremonium</i>
<b>Grain handler's lung</b>	Mouldy grain	<i>Saccharopolyspora rectivirgula</i> , <i>Thermoactinomyces vulgaris</i>
<b>Greenhouse HP</b>	Mouldy soil	Fungi ( <i>Aspergillus sp.</i> , <i>Penicillium sp.</i> , <i>Cryptostroma corticale</i> )
<b>Hot tub HP</b>	Mist from hot tubs	Bacteria ( <i>Mycobacterium avium</i> complex)
<b>Humidifier lung</b>	Mist from standing water	Bacteria ( <i>Thermoactinomyces candidus</i> , <i>Bacillus subtilis</i> , <i>Bacillus cereus</i> , <i>Klebsiella oxytoca</i> ) Fungus ( <i>Aureobasidium pullulans</i> ) Amoebae ( <i>Naegleria gruberi</i> , <i>Acanthamoeba polyhaga</i> , <i>Acanthamoeba castellanii</i> )
<b>Isocyanate HP</b>	Paints, resins, polyurethane foams	TDI, HDI, MDI
<b>Japanese summer-type HP</b>	Damp wood and mats	Fungus ( <i>Trichosporon cutaneum</i> , <i>Cryptococcus albidus</i> , <i>Trichosporon ovoides</i> )
<b>Lifeguard lung</b>	Indoor swimming pool	Bacteria (Endotoxin)

**Table 1.1:** Continued.

<b>Lycoperdonosis</b>	<b>Puff ball spores</b>	<b>Lycoperdon puff balls</b>
<b>Malt worker lung</b>	Mouldy barley	Fungus ( <i>Aspergillus clavatus</i> )
<b>Maple bark HP</b>	Mouldy wood bark	Fungus ( <i>Cryptostroma corticale</i> )
<b>Metalworking fluids HP</b>	Mist from metalworking fluids	Bacteria ( <i>Mycobacterium immunogenum</i> , <i>Pseudomonas fluorescens</i> , <i>Acinetobacter lwoffii</i> )
<b>Mollusc shell HP</b>	Mollusc shell dust	Aquatic animal proteins
<b>Mushroom worker lung</b>	Mushroom compost	Bacteria ( <i>Thermophilic actinomycetes</i> )
<b>Oyster shell lung</b>	Shell dust	Oyster shell proteins
<b>Paprika splitter's lung</b>	Paprika dust	Fungus ( <i>Mucor stolonifer</i> )
<b>Pauli's HP</b>	Laboratory reagent	Pauli's reagent
<b>Peat moss HP</b>	Peat moss	Fungi ( <i>Monocillium</i> sp, <i>Penicillium citreonigrum</i> )
<b>Pituitary snuff user's lung</b>	Heterologous pituitary snuff	Bovine and porcine proteins
<b>Salami worker's lung</b>	Salami seasoning	<i>Penicillium camembertii</i> , <i>Penicillium nalgioiense</i> , <i>Penicillium chrysogenum</i>
<b>Saxophonist's lung</b>	Mouldy reed	<i>Candida species</i>
<b>Sequoiosis</b>	Mouldy wood dust	Fungi ( <i>Graphium species</i> , <i>Pullularia species</i> )

**Table 1.1:** Continued.

<b>Silk production HP</b>	<b>Silk worm larvae</b>	<b>Silk worm larvae proteins</b>
<b>Soy sauce brewer lung</b>	Fermentation starter for soy sauce	Fungus ( <i>Aspergillus oryzae</i> )
<b>Suberosis</b>	Mouldy cork dust	Fungus ( <i>Penicillium frequentans</i> )
<b>TMA HP</b>	Plastics, resins, paints	Trimellitic anhydride
<b>Tobacco worker lung</b>	Mouldy tobacco	Fungus ( <i>Aspergillus sp.</i> )
<b>Tree cutter lung</b>	Wood chips from living maple and oak trees	Fungi ( <i>Penicillium</i> (three species), <i>Paecilomyces sp.</i> , <i>Aspergillus niger</i> , <i>Aspergillus sp.</i> , <i>Rhizopus sp.</i> )
<b>Wheat weevil HP</b>	Infested flour	Wheat weevil ( <i>Sitophilus granarius</i> )
<b>Wine grower's lung</b>	Mouldy grapes	<i>Botrytis cinerea</i>
<b>Wood pulp worker lung</b>	Mouldy wood pulp	Fungus ( <i>Alternaria</i> species)
<b>Wood trimmer lung</b>	Mouldy wood trimmings	Fungus ( <i>Rhizopus</i> species)

**Table 1.1:** Continued.

#### **1.1.4: Host and Environmental Factors:**

The prevalence and incidence of HP seems to be less than 10% in population at risk, in spite of the large number of individuals who have contact with the causative antigens (Bourke *et al.*, 2003; Fink *et al.*, 2005). This may be due to various environmental and host cofactors such as duration and frequency of antigen exposure, the size of the antigenic particle, amount of rainfall, human leukocyte antigen (HLA) type, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) promoter gene polymorphisms and cytokine receptor mutations (Fink, 1992; Schaaf *et al.*, 2001; Schuyler, 2001; Fink *et al.*, 2005). The use of respiratory masks in the work environment may also influence the prevalence, onset and severity of HP (Bourke *et al.*, 2003). It may reduce the level of circulating antibodies because of less exposure to provoking antigens. However, it may be difficult for many people to keep the mask on under strenuous physical activities (Bourke *et al.*, 2003). It has been suggested that intense and intermittent antigen exposure in a susceptible individual usually results in an acute onset of disease whilst prolonged low level exposure causes chronic HP (Kaltreider, 1993; Perez-Padilla *et al.*, 1993; Calvert *et al.*, 1999; Bourke *et al.*, 2001; Irifune *et al.*, 2003). The best example of this can be seen in PFL and budgerigar's lung in the UK. Low grade and prolonged exposure to the antigen tends to lead to the development of the chronic form of HP as mostly seen in budgerigar fanciers (Tanaka *et al.*, 1995; Calvert *et al.*, 1999; Bourke *et al.*, 2003). On the other hand, intermittent high exposure to the bird antigens tends to result in the development of recurrent episodes of acute disease. Acute disease is mostly experienced by pigeon fanciers in the UK who typically keep 100-200 pigeons in a loft (Bourke & Boyd, 1997). This is accompanied with higher specific antibody titres during the pigeon sporting season in late summer and shedding feathers in the autumn (Dalphin *et al.*, 1993; Tanaka *et al.*, 1995; Bourke *et al.*, 2003). A seasonal variation was also suggested to be important in farmer's lung. During rainy summers, stored damp hay

starts moulding which generate heat. This facilitates microbial proliferation and growth of *Thermophilic actinomycetes* and therefore farmer's lung is most commonly seen in late winter (Takahashi *et al.*, 2002; Bourke *et al.*, 2003). Summer type Japanese HP is another example of seasonal variation in the incidence of HP. This form of HP accounts for almost 74% of all HP cases in Japan (Ando *et al.*, 1991; Ohtani *et al.*, 2000) and mainly appears during summer and mid-autumn when fungal growth in older wooden homes is stimulated by warm and moist air (Miyagawa, Hamagami & Tanigawa, 2000).

Furthermore, it has been well established that other cofactors such as the presence of an additional lung inflammation and host smoking habit may also have some effect on the interaction between the relevant antigen and the host immune response (Kalra *et al.*, 2000; Lahmouzi *et al.*, 2000; Al-Ghamdi & Anil, 2007).

Concomitant infection with various micro-organisms may enhance lung responses to the causative antigen in a sensitized subject resulting in the development of HP (Dakhama *et al.*, 1999a; Cormier & Israel-Assayag, 2000). This may explain the reason of developing the disease after having contact with the causative antigen for many years. For instance, Cormier *et al.* (1996) reported that Sendai virus infection in mice exposed to HP antigens or *Saccharopolyspora rectivirgula* may enhance their responses to them (Cormier *et al.*, 1996; Cormier & Israel-Assayag, 2000). They suggested that viral infection is generally the modulator of immune responses, as it will increase the antigen presenting capacity of alveolar macrophages, decreases phagocytosis and antigen clearance, and induces the proliferation of T helper1 lymphocytes. Dakhama *et al.* showed that *Influenza A* viruses were positive in the nasopharyngeal secretions of the majority of patients with HP in their study group (Dakhama *et al.*, 1999b). They suggested the important role of the lung inflammation caused by this virus on development of HP following antigen exposure. Finally additional lung inflammation due to *Mycoplasma pneumoniae*, *Chlamydia psittaci*,

and *Bacille Calmette-Guerin (BCG)* has been also suggested to influence the development of HP (Moore, Hensley & Fink, 1975; McGavin, 1986; Bourke *et al.*, 1992).

Smoking habits of individuals may also influence the host immune response. It has been reported that smoking cigarette influences antigen presentation and antibody production, inhibits lymphocyte (Kalra *et al.*, 2000) and fibroblast proliferation (Lahmouzi *et al.*, 2000) and also compromises the function of natural killer cells (Mian *et al.*, 2007). There is also a decrease in the secretion of interleukin-1 (IL-1), IL-6, IL-12, interferon-  $\gamma$  (IFN- $\gamma$ ) and TNF- $\alpha$  (McCrea *et al.*, 1994; Sauty *et al.*, 1994; Matsunaga *et al.*, 2001). This may reduce antibody responses and affects macrophage function which decreases the risk of HP (Baldwin *et al.*, 1998b; Blanchet, Israel-Assayag & Cormier, 2004).

Host factors may also influence the prevalence, latency and severity of HP (Fink *et al.*, 2005). Overall HP has been reported to be more common in males than females although PFL particularly affects females in Mexico since they keep pigeons in their homes and Mexican females spend more time at home (Perez-Padilla *et al.*, 1993). HP has been diagnosed in all ages including infants and children and 25% of affected children have a positive family history of HP which may indicate a link between genetic predisposition and HP or suggest exposure to the same provoking antigen (Fan, 2002; Ratjen *et al.*, 2003). Furthermore, several studies have indicated that genetic differences between individuals could be important in the occurrence and outcome of disease. The link between a particular HLA and HP has been supported by increased presence of HLA- DR7 in PFL in Mexican population (Selman *et al.*, 1987), HLA- B8 in farmer's lung and PFL in Caucasians (Rodey *et al.*, 1979), HLA- DQw3 in Japanese summer- type HP (Ando *et al.*, 1989), HLA- DRB1\*1305 and HLA-DQB1\*0501 alleles in PFL (Mohr, 2004), and HLA-DRPhe47 in beryllium HP (Amicosante *et al.*, 2005). For instance, Mexican pigeon fanciers with HLA- DR7 tend to develop lung fibrosis with a poor prognosis whilst HP usually has a benign

clinical course in Caucasian populations (Chapela-Mendoza & Selman-Lama, 1999). There is some discussion on the role of genetic factors and the influence of environmental factors in HP. However, it is believed that HLA type may play an important role in binding and presentation of relevant antigen to T cell receptor (TCR) (Sospedra *et al.*, 2006). It has also been suggested that the balance between proteolytic and antiproteolytic processes in the extracellular matrix of an individual's lung is very important and polymorphisms in tissue inhibitors of metalloproteinases 3 (TIMP-3) may influence the susceptibility to idiopathic pulmonary fibrosis and PFL (Hill *et al.*, 2004). Matrix metalloproteinases (MMPs) are highly regulated zinc dependent proteinases with an important role in turnover of the extracellular matrix which is vital in maintenance of the lung structure. They are induced during inflammatory responses and TIMP inhibits these proteinases in order to prevent excessive tissue damage (Ho *et al.*, 2008). Recently it has been shown high levels of TIMP-3 are produced by peripheral blood mononuclear cells of asymptomatic pigeon fanciers (Lee, 2008).

Finally, other combinations of polymorphisms of different genes may also influence the susceptibility to particular diseases. For instance, gene polymorphisms in TNF- $\alpha$  promoter with an increased frequency of TNF-2<sup>-308</sup> allele in patients with HP were reported (Camarena *et al.*, 2001; Schaaf *et al.*, 2001; Schuyler, 2001). Since TNF- $\alpha$  is one of the more important mediators in HP, this polymorphism may be helpful to predict the susceptibility of individuals to HP.

### **1.1.5: Immunopathogenesis:**

HP is defined as an inflammatory interstitial lung disease resulting from hypersensitivity immune reactions against a range of antigens. This is a complex process which involves both arms, cellular and humoral, of the immune response (Kaltreider, 1993).

Initially it was reported that HP was an immune complex mediated disease but subsequently studies have shown that T cells are important in the pathogenesis of disease and it has been suggested that the inflammatory cytokines may have even greater roles to play in the pathogenesis of disease than immune complexes (Moore *et al.*, 1974; Fink, Moore & Barboriak, 1975; Schuyler, Thigpen & Salvaggio, 1978; Curtis *et al.*, 1991; Ohkawa *et al.*, 2001).

In humans following antigenic stimulation of the respiratory tract, foreign antigens are engulfed by alveolar macrophages and subsequently these cells express high levels of class II MHC and the co-stimulatory B7 molecules which helps to transport antigens to the cell surface and to present the complex of MHC/antigen to T helper (Th) cells (Gordon *et al.*, 1995; Kaneko *et al.*, 2000; Desombere *et al.*, 2005). Following interaction between TCR and these complexes, Th cells become activated and produce inflammatory cytokines after interaction with B cells. As a consequence, B cells start dividing and differentiating into plasma cells which secrete antibodies (Jenkins *et al.*, 2001). These interactions are explained in more detail in chapters 4 and 5.

There is a number of animal models of HP using various animal species such as rats, rabbits, guinea pigs, mice, primates and calves (Fink *et al.*, 2005). Schyler et al (1997) were able to adoptively transfer experimental HP by intravenous injection of a T helper 1 (Th1) cell line that produced high levels of IFN- $\gamma$  and low level of IL-4 to naive recipient mice (Schuyler *et al.*, 1997). In a further study the role of cytokines such as IFN- $\gamma$  was



emphasized as an essential factor for the development of granulomatous inflammation in IFN- $\gamma$  (-/-) mice (Gudmundsson & Hunninghake, 1997).

The reaction between antigen and antibody produces immune complexes (IC), which may become deposited in the basement membrane of the lungs and these will then activate the complement cascade by the classical pathway (Cunningham & Quigg, 2005; Wasowska *et al.*, 2007). In addition to this, antigens associated with HP may provoke lung inflammation directly via activating complement through the alternative pathway together with stimulating alveolar macrophages (Berrens, Guikers & van Dijk, 1974; Calvert *et al.*, 1999; Bourke *et al.*, 2001).

Complement activation leads to cell lysis, mast cell activation and neutrophil, monocyte and natural killer (NK) cell recruitment. It has also been suggested that pulmonary macrophages have an important role in the pathogenesis of disease. They secrete IL-8, a chemotactic factor for neutrophils, and macrophage inflammatory protein (MIP)-1 $\alpha$  which is a chemotactic factor for CD8<sup>+</sup> T cells (Denis, 1995; Kurup, Zacharisen & Fink, 2006). Neutrophils attracted to the site of inflammation try to phagocytose the IC deposited in the lung wall and also release lytic enzymes causing localized tissue damage. A transient neutrophilic alveolitis accompanies the symptoms of the acute phase of HP (Reynolds *et al.*, 1993; Razin, Pecht & Rivera, 1995).

Subsequently there is an influx of activated T cells with a predominance of CD8<sup>+</sup> T cells (Kurup, Zacharisen & Fink, 2006) which can modulate granuloma formation. It has been suggested that CD8<sup>+</sup> T cells may have a protective effect on the progress of pulmonary fibrosis (Costabel *et al.*, 1984; Murayama *et al.*, 1993). After long term exposure to the antigen and during the chronic phase of HP, there is an increase in activated CD4<sup>+</sup> T cells (Barrera *et al.*, 2008). These CD4<sup>+</sup> T cells can be divided into Th1 or Th2 subset based on their pattern of cytokine secretion (Irifune *et al.*, 2003). Th1 cells secrete IL-2, IFN- $\gamma$  and

TNF- $\beta$  which help in activating resting macrophages, increase their phagocytic activity and are essential for development of disease (Nance *et al.*, 2005). Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 which can activate B cells to produce antibodies and promote mast cell maturation and proliferation (Abbas, Murphy & Sher, 1996). Regulatory cytokines such as IL-10 may down regulate the inflammatory responses (Mosmann *et al.*, 1986; Schuyler *et al.*, 1997). During the chronic stage of HP the levels of IL-6 and TNF- $\alpha$  are higher as compared to the acute stage which emphasizes the importance of these cytokines in the development of pulmonary fibrosis (Needleman, Wigley & Stair, 1992; Ohtani *et al.*, 1999). On the other hand, the release of lytic enzymes from activated macrophages can cause a non-specific tissue damage and formation of granulomatous lesions (Squier & Cohen, 1994; Kinet, 1999).

Further studies have been carried out in HP to understand the pathogenesis of disease. The presence of IgM rheumatoid factor (RF) is a frequent finding in HP (Martinez-Cordero, Negrete-Garcia & Mendoza, 1992; Aguilar Leon, Novelo Retana & Martinez-Cordero, 2003; Araiza *et al.*, 2007) suggesting IgM RF producing B cells may influence T cell activation and induce antibody differentiation and isotype switching of immunoglobulins (Araiza *et al.*, 2007). High levels of nerve growth factor (NGF) have been found in culture of lymphocyte from pigeon fanciers. NGF can be secreted by lymphocytes and mast cells which shows a potential route between airway inflammation and production of this factor (McSharry *et al.*, 2006a).

Furthermore, the tracheobronchial mucociliary system is also very important in the clearance of inhaled antigens and it has been suggested that mucociliary clearance is compromised in symptomatic pigeon fanciers (Hasani *et al.*, 1992). Finally, other cell populations have also been recovered from bronchoalveolar lavage (BAL) fluid in patients with PFL. Natural killer T (NKT) cells were recovered in increased numbers in BAL fluid

which are able to induce Th1 mediated immune response (Korosec *et al.*, 2007) and also high numbers of  $\gamma\delta$ -T cells have been recovered in BAL fluid in PFL although their roles are not fully understood (Raulf *et al.*, 1994).

In summary, HP is a complex disease and both cellular and humoral aspects of immune system are involved in the immunopathogenesis of disease. T cells, macrophages and NK cells together with different inflammatory cytokines and specific antibodies may contribute to protective immunity. However, many unsolved problems remain to be investigated in the pathogenesis of HP.

#### **1.1.6: Clinical Presentation:**

The clinical features of HP depend on the antigenicity and size of the antigen together with the frequency and intensity of antigen exposure and the amount of immunological response of host (Kaltreider, 1993; Calvert *et al.*, 1999; Bourke *et al.*, 2001; Irifune *et al.*, 2003). Clinically HP can be classified as acute, subacute and chronic disease.

Acute disease has a recurrent episodic nature and usually occurs within 4 to 8 hours after high level of exposure to the relevant antigen in a susceptible individual (Kaltreider, 1993). A flu-like syndrome starts abruptly with fever, chills, malaise, myalgia, severe dyspnoea, chest tightness and dry cough. Findings during physical examination of patients consist with fever, tachypnoea, cyanosis and basilar crackles in the lungs (Kaltreider, 1993; Bourke *et al.*, 2001). Between attacks the patient may be completely normal (Kaltreider, 1993; Kurup, Zacharisen & Fink, 2006).

The subacute form of HP is due to low level of exposure to the antigen and is more insidious. The progressive respiratory symptoms develop over weeks with hypoxemia and crackles in the lungs (Kurup, Zacharisen & Fink, 2006).

Chronic HP usually occurs as a result of continuous and prolonged low level exposure to the provoking antigen resulting in progressive, irreversible and debilitating fibrotic lung disease in susceptibility individuals with insidious onset (Kaltreider, 1993; Irifune *et al.*, 2003). As a consequence of interstitial inflammation and lung fibrosis the patient develops increasing dyspnoea, fatigue, anorexia, cough and general weight loss. During physical examination restricted ventilation, basilar crackles in the lungs and signs of right sided heart failure (Bourke *et al.*, 2001; Patel, Ryu & Reed, 2001; Schuyler, 2002) may be seen.

#### **1.1.7: Laboratory Features:**

Routine laboratory tests for HP are often non-specific and no single laboratory test is diagnostic. There may be a neutrophilic alveolitis during acute episodes of disease (Navarro *et al.*, 2006; Yoshikawa *et al.*, 2006), whilst the bronchoalveolar lavage (BAL) fluid may be normal in chronic disease (Mohr, 2004). There is also a polyclonal increase in immunoglobulin level in HP, as shown by serum protein electrophoresis (Kaltreider, 1993).

It has been reported that KL-6 is significantly higher in the serum samples of individuals with PFL (McSharry *et al.*, 2006b). KL-6 is a high-molecular-weight sialoglycoprotein secreted by proliferating alveolar type 2 epithelial cells which indicate lung inflammation in interstitial lung disease.

#### **1.1.7.1: Serologic Studies:**

Specific serum precipitating IgG antibodies against the provoking antigen indicates intense exposure and are usually detectable in individuals with HP (Lacasse *et al.*, 2003; Morris, 2003; Kampfer *et al.*, 2005; McSharry *et al.*, 2006b). IgG antibody against pigeon serum antigens has been quantified in pigeon fanciers by automated fluorimetry and levels above 10 mg/L were associated with disease (McSharry *et al.*, 2006b). However, precipitating antibodies are also present in 50% of asymptomatic susceptible individuals exposed to the relevant antigen (Kaltreider, 1993; Baldwin *et al.*, 1998c).

A positive Arthus Reaction can also be induced by a subcutaneous injection of the purified antigen which indicates the sensitization of B cells and T cells (Kaltreider, 1993; Calvert *et al.*, 1999).

Another serological finding is positive rheumatoid factor in PFL which seems to be the result of hypergammaglobulinemia and immune complex formation (Roosnek & Lanzavecchia, 1991). Positive RF reported in 52.8% of individuals with PFL and 4.2% of asymptomatic pigeon fanciers which can be useful together with high anti-avian antibodies in differentiating the diagnosis of disease (Aguilar Leon, Novelo Retana & Martinez-Cordero, 2003). A possible pathogenic role of RF in HP has been suggested with immune complex formation and complement activation enhancing the inflammatory reaction (Leon *et al.*, 2002).

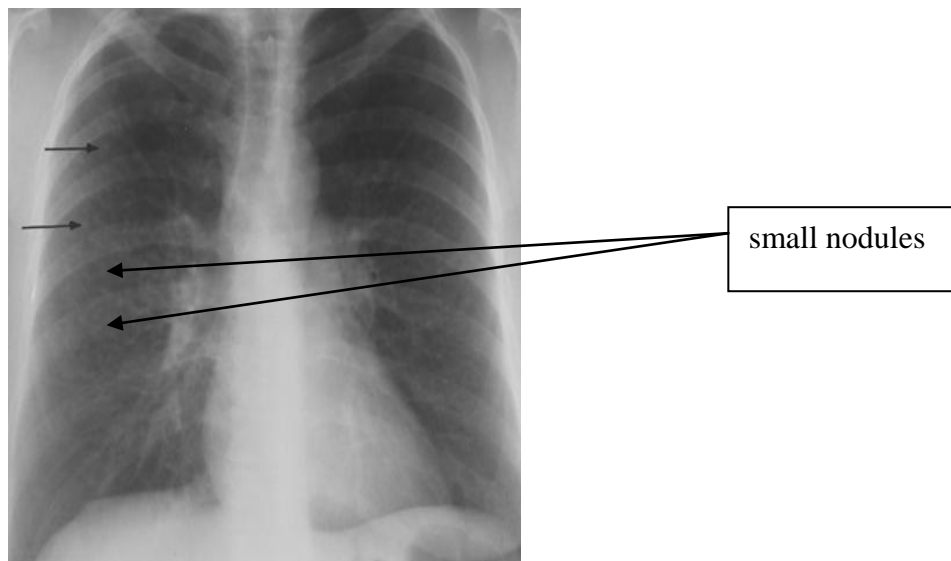
#### **1.1.7.2: Radiological Findings:**

A chest radiograph in acute HP may be either normal or reveal various degrees of reticulonodular infiltration and consolidation and ground glass attenuation at the middle and base of both lungs (Fink, 1992). Figure 1.1 shows patchy airspace consolidation throughout both lungs in a case of acute HP.

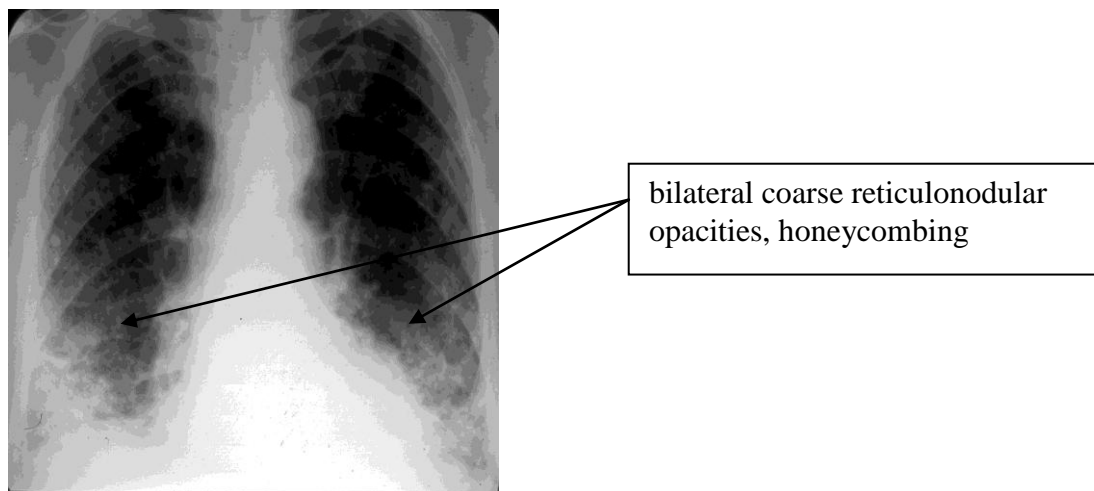
A chest radiograph in chronic HP shows irregular linear opacities, bronchiectasis, bronchitis, diffuse interstitial fibrosis and honey comb appearance in 50% of the cases and progressive reduction in lung volume (Bourke *et al.*, 2003; Fink *et al.*, 2005). Emphysema is another finding in chronic HP and it has been suggested that proteolytic enzymes produced by pathogens in organic dust may be a cause for development of the disease (Erkinjuntti-Pekkanen *et al.*, 1998). Figure 1.2 shows a chest X-Ray of a chronic HP with honey comb pattern and lung volume reduction in the base of both lungs.

High resolution computed tomographic scanning (HRCT) of the chest in acute HP is a sensitive method for the early detection of interstitial changes (Muller & Miller, 1990). Figure 1.3 shows patchy areas of consolidation and ground-glass attenuation in both lungs in acute HP.

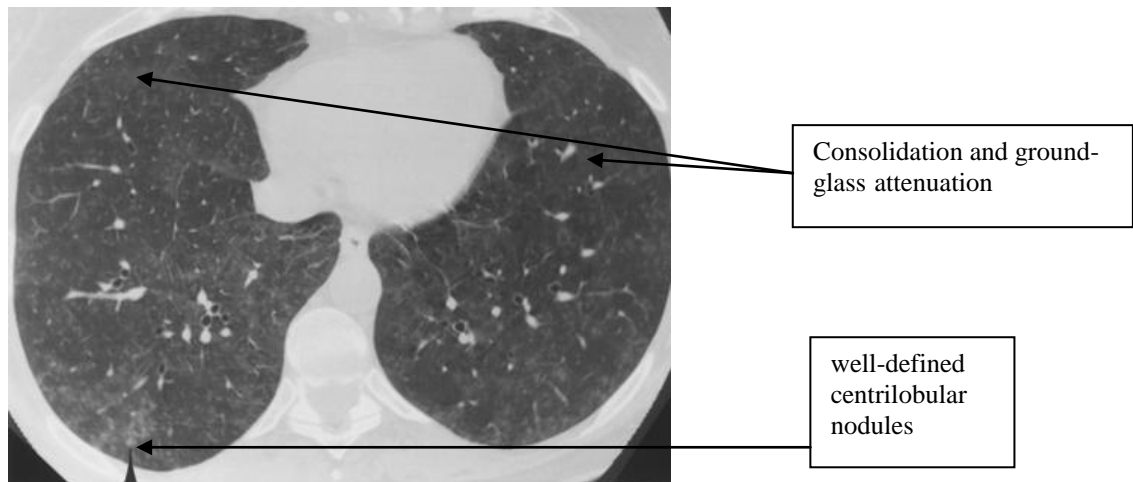
Micronodular infiltration and ground glass opacifications with interlobular septal thickening together with lobular areas with decreased attenuation, vascularity, centrilobular nodules, and absence of abnormalities in lower zone in HRCT represent a chronic type of HP (Silva *et al.*, 2008) (Figure 1.4).



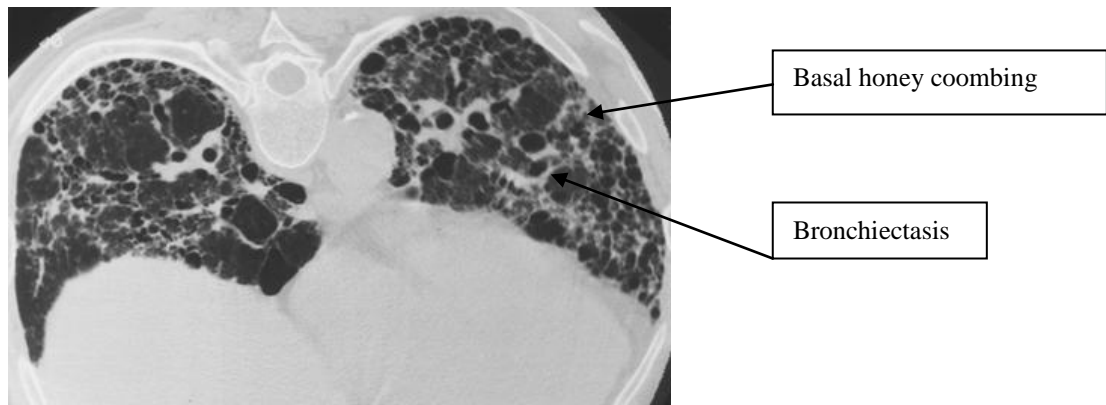
**Figure 1.1:** Acute hypersensitivity pneumonitis in a 36-year-old woman who presented with severe cough and dyspnoea. Chest radiograph shows diffuse small nodules (*arrows*) and normal lung volumes (Matar, McAdams & Sporn, 2000)



**Figure 1.2:** Chest radiograph of a chronic type of hypersensitivity pneumonitis with lung fibrosis. Honey comb pattern and lung volume reduction in the base of both lungs (Matar, McAdams & Sporn, 2000)



**Figure 1.3:** High-resolution CT (HRCT) in a 40 years old woman with pigeon fanciers' lung; scan shows patchy areas of consolidation and scattered ground-glass attenuation in both lungs and well-defined centrilobular nodules (Matar, McAdams & Sporn, 2000).



**Figure 1.4:** HRCT in chronic HP shows basal honeycombing, traction bronchiectasis and architectural distortion (Matar, McAdams & Sporn, 2000).



#### **1.1.7.3: Pulmonary Function:**

Pulmonary function tests may show a restrictive pattern of disease with reduced lung compliance in HP. This test reveals a decreased lung volume, vital capacity, and total lung capacity and an elevated residual volume (Bourke *et al.*, 2003). There is also a reduction in transferring of carbon monoxide (TL<sub>CO</sub>) in the lungs which is a sensitive measurement in detecting acute HP (Kokkarinen, Tukiainen & Terho, 1993; Bourke *et al.*, 2003). Moderate hypoxia and mild hypocarbia may be detectable during the acute presentation of HP but return to normal between acute episodes (Kaltreider, 1993). Another measurement that can be useful in detection of lower respiratory tract inflammation and alveolitis is measurement of exhaled nitric oxide (NO) concentration which increases in alveolitis (Lehtimäki *et al.*, 2001).

In chronic disease, after establishment of interstitial fibrosis, the abnormalities in pulmonary function test are progressive and show a severe restrictive pattern (Allen, 1976).

#### **1.1.7.4: Broncho-alveolar Lavage:**

BAL may help to reveal the presence of lymphocytic and neutrophilic infiltrations of the interstitium and alveoli in HP. It has been reported that the presence of 30% lymphocytes in the BAL fluid cell profile of non-smokers and 20% in smokers can be used as a diagnostic sign in individuals with a history of exposure to provoking antigens (Sterclova *et al.*, 2006).

Analysis of BAL fluid in pigeon breeders 6 hours after inhalation challenge with pigeon antigen (pooled pigeon serum) revealed a significant increase in neutrophils (Reynolds *et al.*, 1993; Ohtani *et al.*, 2000). Neutrophil number decreases with time and they are gradually replaced by lymphocytes (Kurup, Zacharisen & Fink, 2006). Up to 80% of the cells recovered by BAL are lymphocytes with majority being CD8<sup>+</sup> T cells in acute HP, causing an inversion of the CD4<sup>+</sup>/CD8<sup>+</sup> ratio (Pardo *et al.*, 2000; Kurup, Zacharisen & Fink, 2006). The CD4<sup>+</sup>/CD8<sup>+</sup> ratio in normal lung varies by age, for instance the ratio is 0.7 in children at 3-16 years old, 2.7 in young adult and 7.6 at age of 64-83 (Grigg & Riedler, 2000). Different pulmonary diseases may have different CD4<sup>+</sup>/CD8<sup>+</sup> ratios. For instance the CD4<sup>+</sup>/CD8<sup>+</sup> ratio is elevated in sarcoidosis (Reichenberger *et al.*, 2007), bronchiectasis due to mustard gas inhalation (Emad & Emad, 2007) and idiopathic pulmonary fibrosis (Tabuena *et al.*, 2005), and lower in asthma (Marguet *et al.*, 1999).

Sterclova M *et al* (2006) reported that there is a correlation between the continuous and intense exposure to the antigenic dusts in HP and the low CD4<sup>+</sup>/CD8<sup>+</sup> ratio in BAL fluid. This could be due to the protective effect of CD8<sup>+</sup> T cells against pulmonary fibrosis since it is believed that CD4<sup>+</sup> T cells may have an important role in the pathogenesis of pulmonary fibrosis (Murayama *et al.*, 1993; Sterclova *et al.*, 2006).

Recently NKT cells have been identified as a unique subset of T cells with immunoregulatory function (Korosec *et al.*, 2007) and they have been recovered from BAL

fluid of patients suffering from HP suggesting these cells may be involved in the pathogenesis of HP (Korosec *et al.*, 2007). They produce large amount of various cytokines including IL-4 and IFN- $\gamma$ , and it has suggested that they play an important role in the induction of protective CD8<sup>+</sup> T cells and the homeostatic proliferation of these cells (Ueda *et al.*, 2006).

#### **1.1.7.5: Inhalation Provocation Studies:**

Inhalation provocation studies are a procedure used to definitively establish the role of a particular causative antigen in HP (Hage-Hamsten *et al.*, 2007). This challenge usually induces a transient inflammation without long term complications and has been used for clinical and research purposes in the past by experienced investigators (Fink *et al.*, 2005). Safety precautions are important and the challenge with suspected antigen is conducted at the hospital, laboratory or workplace.

A highly purified suspension of suspected antigen is given as an inhalation challenge in order to limit the chance of any nonspecific irritant contamination to minimum. The clinical and laboratory responses such as blood count, PaO<sub>2</sub> and pulmonary function tests are recorded. The reproduction of the signs and symptoms of acute HP in about 4-6 hours after the challenge should be considered as a positive response and this proves a strong relationship between the suspected antigen and acute HP, suggesting the important role of immune complexes, complement and neutrophils during disease (Kaltreider, 1993).

For instance, pooled pigeon sera has been used by Ramirez-Venegas *et al* (1998) for inhalation challenge in an open space (hospital yard) through a jet nebulizer (Ramirez-

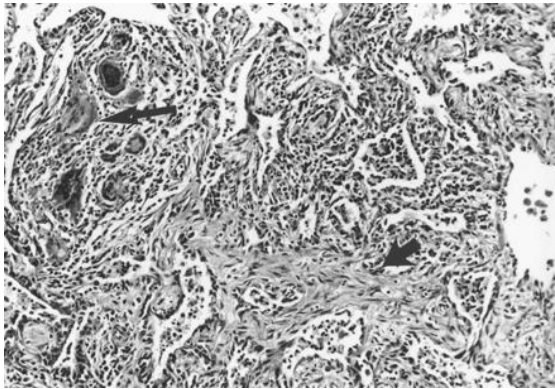
Venegas *et al.*, 1998). Also Ohtani et al (2000) have used fresh avian droppings extracts to carry out inhalation test through a hand nebulizer for their study subjects (Ohtani *et al.*, 2000). Inhalation provocation tests have also been useful in other diseases such as asthma (Brannan *et al.*, 2005).

#### **1.1.7.6: Lung Biopsy:**

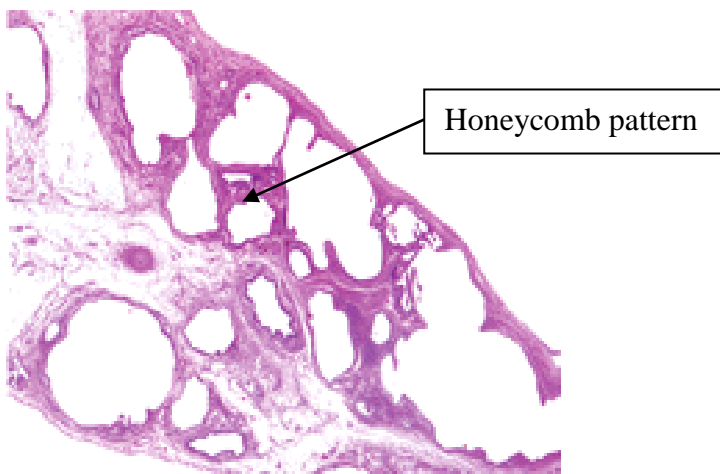
Lung biopsies can be carried out either as transbronchial (through bronchus) biopsy or open lung biopsy, nevertheless requesting an open lung biopsy must be balanced against a patient's morbidity (Lacasse & Cormier, 2006).

Acute HP is characterised by infiltrations of lymphocytes, plasma cells and polymorphonuclear leukocytes in alveolar walls together with oedema and poorly formed granuloma (Perez-Padilla *et al.*, 1993; Fink *et al.*, 2005; Lacasse & Cormier, 2006) (Figure 1.5).

A lung biopsy in a chronic HP reveals intense peribronchiolar lymphocytic infiltration, plasma cells and foamy macrophages in the alveoli and interstitial spaces. This may be accompanied by non-caseating granulomas, bronchiolitis, emphysema and lung fibrosis (Madison, 2008) (Figure 1.6).



**Figure 1.5:** Microscopic features of acute HP: interstitial inflammation accompanied by pneumonia (*thick arrow*) and multinucleate giant cell (*thin arrow*) which is typical of HP (Matar, McAdams & Sporn, 2000).



**Figure 1.6:** Microscopic features of chronic HP: biopsy specimen shows honeycomb pattern and moderate mononuclear interstitial infiltrate (Silva, Churg & Muller, 2007).

### **1.1.8: Diagnosis:**

Clinical features and laboratory tests are not diagnostic for HP and the diagnosis of HP is usually established by a combination of clinical features, history of contact with provoking antigen, and exclusion of other similar conditions. The first step is having a strong suspicion of an environmental antigen being associated with disease. A detailed and accurate history of occupational or environmental exposure to the potential provoking antigens may suggest the aetiology of the disease (Kaltreider, 1993). However, it may be difficult to differentiate the acute type of HP from other respiratory diseases triggered by allergens or toxic organic dusts (Bourke *et al.*, 2003).

Diagnosis cannot be established based on single clinical feature or laboratory test and a combination of them together with radiographic changes must be used (Bourke *et al.*, 2001; Lacasse *et al.*, 2003). Schuyler M et al (2002) described 6 major criteria and 3 minor criteria with the diagnosis of HP being made in patients fulfilling 4 major criteria together with 2 minor criteria (Schuyler, 2002).

The major criteria are:

1. Developing symptoms compatible with HP following exposure to provoking antigen.
2. Confirmation of exposure to provoking antigen by history, positive specific precipitating antibodies and or BAL specific antibodies.
3. Compatible chest radiograph or CT scan changes.
4. Lymphocytosis observed from BAL fluid.
5. Compatible histological changes in lung biopsy.
6. Positive inhalation provocation test.

The minor criteria consist:

1. Basilar crackles in chest auscultation.
2. Reduced diffusion capacity.
3. Arterial hypoxemia.

However, establishing a diagnosis, especially in chronic HP in which the environmental offending antigen is not suspected and the patient's condition deteriorates very slowly, may be a puzzle. In this situation a histological examination can provide some clue as to any further investigations required to establishing the diagnosis (Bourke *et al.*, 2001; Schuyler, 2002; Lacasse *et al.*, 2003).

#### **1.1.9: Therapy:**

The most important aspect in the management of HP is the avoidance of the provoking antigen (Mohr, 2004; Venkatesh & Wild, 2005) with education being useful in the prevention of respiratory problems (Kaltreider, 1993; Kurup, Zacharisen & Fink, 2006). Unfortunately due to the financial and emotional commitment in some of the occupations or hobbies, individuals are reluctant to seek medical advice at an early stage. For instance, farmers should be educated about the risks of exposure to barns dust and preventive measures such as wearing a mask and better ventilation in the barn should be considered (Lacasse & Cormier, 2006). Also in pigeon fanciers, reducing the time spent with the birds, wearing suitable mask, hat and coat and ventilation measurements may be beneficial (McSharry, Anderson & Boyd, 2000). Other sources of exposure to the bird antigens (duvets, pillows) should also be considered (Inase *et al.*, 2003). The antigenic dusts from

pigeon lofts can persist for 18 months after ceasing contact with birds which may even sensitise other family members or children (McSharry, Anderson & Boyd, 2000).

A short course of treatment with corticosteroids such as prednisolone 0.5-1 mg/Kg/day or 40 mg/day for 2-3 weeks can cause a rapid improvement in lung function in patients with acute episodes of disease (Kokkarinen, Tukiainen & Terho, 1992; Mohr, 2004; Venkatesh & Wild, 2005). Acute HP has also successfully been treated in a mushroom farm worker with an extra-fine aerosol corticosteroid (beclomethasone dipropionate (BDP) dissolved in hydrofluoroalkane-134a (HFA) (Tanaka *et al.*, 1995).

Corticosteroids inhibit Th2 cytokine synthesis and also enhance IL-10 production by T cells and airway cells which can inhibit IFN- $\gamma$  production by CD4<sup>+</sup> T cells (Richards *et al.*, 2000).

In chronic HP, treatment is supportive but higher doses of corticosteroids for a longer duration may be needed; however, the effectiveness of corticosteroids in long-term prognosis is still under debate (Lacasse & Cormier, 2006).



## **1.2: Pigeon Fanciers' Lung:**

### **1.2.1: Introduction:**

PFL is a form of bird breeders' lung which was first described by Reed et al in the mid 1960s (Reed & Barbee, 1965). A large variety of birds may cause bird breeders' lung and among them pigeons, lories, budgerigars, canaries, lovebirds, mynas, macaws, parakeets and parrots are the more common (Kurup, Zacharisen & Fink, 2006). PFL is one of the most common types of HP which is caused by the repeated inhalation of pigeon antigens in a sensitised subject (Bourke & Boyd, 1997).

Pigeon racing is a competitive sport with large financial prizes and to be successful an individual needs to make a large financial and emotional commitment to this sport (Bourke & Boyd, 1997; McSharry, Anderson & Boyd, 2000). Pigeon fanciers spend extensive amounts of their time and money in order to train and breed their pigeons and such fanciers may be reluctant to cease their contact with the birds when symptoms start to appear since it is a way of life rather than just a hobby (McSharry, Anderson & Boyd, 2000). Exposure to pigeon antigens mostly happens while individuals spend times inside pigeon lofts and train their pigeons during the summer racing season (Calvert *et al.*, 1999; McSharry, Anderson & Boyd, 2000). However, antigenic dust may be transferred to the home environment from the loft by hair and clothing of the fanciers and subsequently affects other family members (McSharry, Anderson & Boyd, 2000).

### **1.2.2: Antigen Exposure in Pigeon Loft:**

Antibodies against different sources of antigen in pigeons have been identified. Pigeon droppings, feathers, serum, egg yolk and white and gut wall contain a mixture of proteins, glycoproteins and polysaccharides which can be the sources of antigen (McSharry, Anderson & Boyd, 2000).

The dust inside the pigeon loft contains desiccated pigeon droppings, feather dust (pigeon bloom as shown in figure 1.7) and the dust from grain feed. Routinely pigeon fanciers spend 20 hours or more per week in their lofts. The loft might be cleaned out daily, therefore, it does not generate great amounts of dust and in this situation pigeon feathers produce the most dust. On the other hand, the pigeon droppings might be accumulated on the floor as an absorbent for fresh droppings which is called the deep litter system as shown in figure 1.8 (McSharry, Anderson & Boyd, 2000). This can be a source of substantial amount of dust.

Initially an acidic glycoprotein with a 200 KDa weight was extracted from pigeon droppings and identified as pigeon IgA (Edwards, Fink & Barboriak, 1969; McSharry, Anderson & Boyd, 2000) and it was suggested that this was the major pigeon antigen in PFL and is present in both pigeon bloom and droppings. Rodrigo et al (2000) compared the results of an inhibition ELISA for pigeon serum against bloom and they concluded that pigeon bloom is a stronger antigenic source than pigeon serum (Rodrigo *et al.*, 2000).

Pigeon bloom is a fine powder with a waterproofing nature on the flight feathers and is produced in copious quantities by healthy birds. These particles are one to three microns in size and contain the flattened squamous keratinised pigeon epidermal cells. Keratin is a highly hydrophobic protein and very resistant to enzymatic degradation. The bloom may act both as a carrier to take soluble antigens to the lower airways and as an irritant and foreign body causing granuloma in the lung (Longbottom, 1989; McSharry, Anderson &

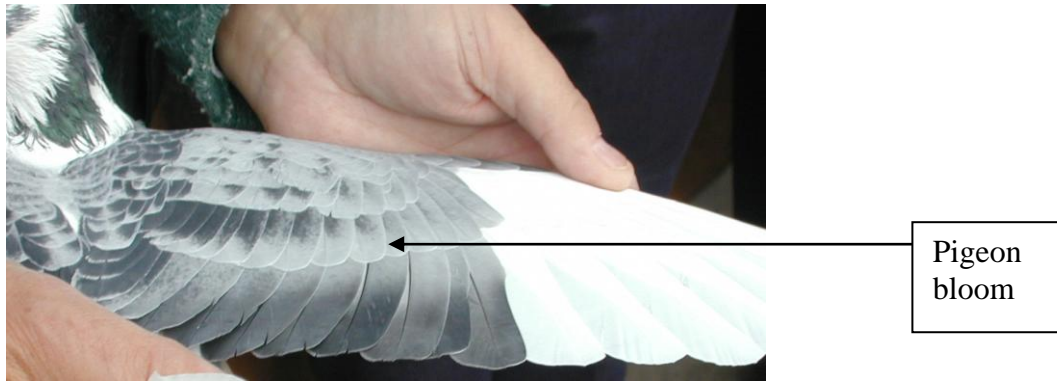
Boyd, 2000). It can be deposited on hands and clothing of the fanciers. Therefore, pigeon bloom has been suggested as having high levels of antigenic potential (Baldwin *et al.*, 1998a; McSharry, Anderson & Boyd, 2000).

In addition to sources described above, pigeon dropping extract and pigeon bloom also contain a large glycoprotein identified as pigeon intestinal mucin (PIM) (Fredricks, 1978). Mucin has a high carbohydrate content which has been reported to be disease-associated and relatively resistance to biochemical breakdown (Todd, Coan & Allen, 1991; Baldwin *et al.*, 1998a; Baldwin *et al.*, 1999; Thornton & Sheehan, 2004). PIM can stimulate very high antibody titres in pigeon fanciers and it has been suggested to be a major antigen in PFL (chapter 4) (Todd, Coan & Allen, 1991; Baldwin *et al.*, 1998c).

Although fungal spores have not been implicated in pigeon breeder's disease, pigeon feather mites; *Diplaegidia columbae*; may induce IgE antibody responses and these can be associated with significant asthma-like symptoms which can contribute to the symptoms of the disease (Colloff *et al.*, 1997; McSharry, Anderson & Boyd, 2000). Finally, bacteria, fungi and their products may be spread in pigeon droppings (McSharry, Anderson & Boyd, 2000). Therefore, the dust of the pigeon lofts will contain various bacterial and fungal products such as bacterial lipopolysaccharide, teichoic acid and fungal beta-glucan which may damage the lung tissue in fanciers (Cuthbert & Jeffrey, 1995; McSharry, Anderson & Boyd, 2000). Microorganisms that have been detected in pigeons or pigeon droppings are *Chlamydia*, *Mycoplasma*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Influenza A* and *B* (Springston, 1998; McSharry, Anderson & Boyd, 2000) and it has been suggested that co-infection with one or more of these organisms may influence the development of disease (Cormier *et al.*, 1994; Dakhama *et al.*, 1999b).

Some of these antigens are highly resistant to degradation resulting in the production of persistent immune complexes which could be difficult to be cleared off the system,

therefore contribute to greater damage to the lungs (Bourke *et al.*, 1990). Exposure to these antigens can start a series of pathological events which lead to the alveolitis together with an increase in the permeability of the alveolar capillary membrane in sensitised fanciers (section 1.1.5).



**Figure 1.7:** Pigeon Bloom (Photo curtesy of Dr CI Baldwin).



**Figure 1.8:** Deep litter system in pigeon loft (Photo curtesy of Dr CI Baldwin).

## **2: Hypothesis and Aims**

### **2.1: Hypothesis:**

Although many studies have been carried out on PFL, there are still many questions unanswered as to the underlying mechanisms of disease. Generally it is considered that the acute stage of disease is caused by immune complexes (Fink, Moore & Barboriak, 1975; Yoshizawa *et al.*, 2001; Kurup, Zacharisen & Fink, 2006).

Therefore the starting point for PFL is the generation of immune complexes. However, this does not explain why some fanciers are asymptomatic despite the presence of high levels of anti-avian antigen antibodies in their serum and why some symptomatic fanciers have low levels of these antibodies.

The IgG subclass composition of the immune response to respective antigens is important in the composition of immune complexes and may influence the induction and development of disease. This IgG subclass distribution in the immune complexes will depend on both the quality and the functional affinity of each isotype.

It has been shown that anti-mucin IgG1 responses have an important role in the development of disease although why different fanciers make such subclass responses is unknown. IgG1 is considered to be a T-dependent (TD) antibody and thus it has been suggested that T cell responses to mucin may also be important in the development of disease.

Thus the hypothesis for this study is that pigeon anti-mucin IgG and T cell responses are critical in the development of PFL.

## **2.2: Aims:**

The aims of this study were:

- 1) To study antibody responses in symptomatic and asymptomatic pigeon fanciers and to identify the functional affinity of specific anti-pigeon antigen IgG isotypes.
- 2) To generate mucin-specific T cell clones from pigeon fanciers and to identify the T cell phenotype, cytokine profile and the antigen and epitope specificity of these cells.

Therefore the objectives of this study were:

- 1) Preparation of pigeon intestinal mucin from pigeon intestines.
- 2) Quantification of the IgG and IgG subclasses against a series of pigeon antigens including pigeon serum (PS), PIM, trypsinised mucin (TM, t mucin), old pigeon droppings (PDO), fresh pigeon droppings (PDF) and pigeon intestinal scrapings (PIS).
- 3) Comparison of these antibody responses between symptomatic and asymptomatic pigeon fanciers.
- 4) Determination of the functional affinity of IgG subclasses in symptomatic and asymptomatic fanciers by chaotropic inhibition ELISA and isothermal microcalorimetry (ITC).
- 5) Generation of EBV transformed B cell line (feeder cells) from pigeon fanciers.
- 6) Generation of T cell clones from pigeon fanciers.
- 7) Identification of antigen and epitope specificity, phenotype and cytokine profile of T cell clones generated from pigeon fanciers.

### **3: Materials and Methods:**

#### **3.1: Preparation of Pigeon Intestinal Mucin:**

To prepare PIM, the intestines of freshly killed pigeons were washed carefully with water and the mucus gel that covers the mucosal surface of the intestines was scraped with a glass slide into phosphate buffer (PH: 6.5) containing the proteinase inhibitors (1 mM phenyl-methyl-sulphonyl-fluoride, 5mM EDTA and 5mM N-ethylmaleimide) (Mantle & Allen, 1981; Todd, Coan & Allen, 1991). The mixture was homogenised in a blender for 1 minute, then centrifuged at 10000g for 1 hour. The supernatant (soluble mucus) was stored at -70° C in small aliquots and used as pigeon intestinal scrapings (PIS) in ELISA.

The soluble mucus was also fractionated by caesium chloride (CsCl) density gradient centrifugation for 48 hours at 40000g (starting density: 1.42 g/ml) (Hutton DA, Clin Sci 1990; 78). 9 fractions were collected and 1 ml of each fraction was weighed to determine the density of each fraction. Following 24 hours dialysis in water (minimum 8 changes), the carbohydrate (by PAS method) and protein (by Bio-Rad method) concentration of each fraction were determined (Mantle & Allen, 1978).

The fractions with a higher content of carbohydrate (density: 1.47-1.58 g/ml) were pooled together and fractionated on a second CsCl density gradient (starting density: 1.47g/ml). 9 equal fractions were collected again and the density, carbohydrate and protein contents of each were measured as previously described. The fractions with the highest amount of carbohydrate (density: 1.43-1.47 g/ml) were pooled as PIM and stored at -80° C. Samples were also freeze dried for further use.



### **3.2: Preparing the Trypsinised Mucin:**

To proteolytically digest the sparsely glycosylated region of the protein core of mucin, trypsin (Sigma, UK) was added to PIM at a ratio of one part trypsin to 100 parts mucin in PBS. This mixture was incubated at 37° C for 24 hours (professor J Pearson personal communication). Subsequently, one mole of phenyl methyl sulfonyl fluoride (PMSF, Sigma, UK) was added to each mole of trypsin to inhibit the reaction of the enzyme. Finally this mixture was aliquoted and stored at – 20° C.

### **3.3: Preparing Pigeon Droppings:**

Fresh (freshly voided and green) and old (dry and dusty) pigeon droppings were collected from a pigeon loft. They were vigorously stirred in 200 ml of 1% normal saline/ 1% phenol for 6 hours at room temperature and subsequently centrifuged at 900 g. The supernatant was dialysed against distilled water and freeze dried (Berrens & Maesen, 1972; Baldwin *et al.*, 1998a).

A proportion of these freeze-dried extracts were reconstituted in deionised water to make PDF and PDO at 1 mg/ml concentration for further experiments.

### **3.4: Pigeon Serum:**

Lyophilised PS (Sigma, UK) was reconstituted at 5 mg/ml with distilled water, aliquated in 1 ml volumes and stored at - 80° C for subsequent experiments.

### **3.5: Periodic Acid Schiff's for Carbohydrate Estimation:**

Periodic acid schiffs (PAS) method was used to measure the carbohydrate content of PIM and other pigeon antigens (Mantle & Allen, 1978).

This test was carried out in a 96 well plate (Maxisorp, Nunc, Denmark). A range of standards of papain digested pig mucin (Todd, Coan & Allen, 1991) (A kind gift from Professor Jeff Pearson, Newcastle University) was prepared in PBS at concentrations 70 µg/ml to 10 µg/ml.

200 µl of the standards were added to the plates and the pigeon antigens were double diluted across the plate into PBS.

Subsequently, 10 µl of solution 1 [10 µl of 50% periodic acid (BDH, Bath, UK) in 5 ml of 7% acetic acid (BDH, Bath, UK)] was added to each well and the plate was incubated at 37° C for one hour.

10 µl of solution 2 [0.05 g of sodium metabisulfate (Sigma ,UK) in 3 ml of schiffs reagent (BDH, Bath, UK)] was then added into the wells and the plate was incubated at room temperature for a further 30 minutes. Finally, the plate was read on an ELISA Plate Reader (Bio-TEK, Vermont, USA) at 570 nm. The concentration of the unknowns was read from the standard curve.

### **3.6: Bio-Rad Technique for Protein Estimation:**

The protein content of pigeon antigens were measured by the BioRad technique (Hempstead, UK). To begin with, bovine serum albumin (BSA) (Sigma, UK) was made up at a range of concentrations from 5µg/ml to 100µg/ml in distilled water.

200 µl of each concentration of BSA were placed into a 96 well plate (Maxisorp, Nunc, Denmark) in duplicate. 200 µl of the samples were double diluted across the plate into

distilled water. Finally, 25 µl Bio-Rad Dye Reagent was added to each well and the plate was incubated at room temperature for 5-30 minutes. The plate was read on ELISA Plate Reader (Bio-TEK, Vermont, USA) at 570 nm. The concentration of unknowns was read from the standard curve.

### **3.7: Human Subjects:**

Approval for this project had been obtained from Ethical Committee of Joint Newcastle Health Authority in January 1994. Serum samples had previously been collected from pigeon fanciers who had participated in pigeon shows between 1997 until 2002. Plasma had also been collected from individuals who had no contact with pigeons or any other birds.

The sera had been tested for precipitating antibodies to pigeon serum and pigeon droppings by counter current immunoelectrophoresis (CIE) and the sera had been considered positive when the precipitins were present to both pigeon serum and droppings (Baldwin *et al.*, 1998c). A clinical questionnaire had also been completed at the same time with information about the nature, frequency and severity of the symptoms together with the background details such as degree of pigeon contact and history of smoking being collected. A clinical diagnosis was made based on the presence of at least one respiratory symptom such as dry cough and at least one systemic symptom such as fever, following pigeon contact on at least 3 separate occasions.

Based on the clinical findings and IgG titres of equal or greater than 10000, human subjects were classified into 4 clinical groups (A-D). One additional group was also

allocated as control negative group consisting individuals who did not have any contact with pigeons. These groups were:

- Group A: Symptomatic with ELISA titres  $\geq 10000$  (n: 25)
- Group B: Asymptomatic with ELISA titres  $\geq 10000$  (n: 25)
- Group C: Symptomatic with ELISA titres  $< 10000$  (n: 25)
- Group D: Asymptomatic with ELISA titres  $< 10000$  (n: 25)
- Group E: Control negative with no pigeon contact and ELISA titres  $< 1000$  (n: 10)

The serum samples were aliquoted and stored at  $-80^{\circ}\text{C}$ . Data was coded for confidentiality and stored on Microsoft Office 2003.

Peripheral blood mononuclear cells were separated from fresh blood taken from a number of asymptomatic pigeon fanciers (group B) for T cell cloning.

### **3.8: ELISA Checker Board Analysis:**

Optimal conditions and dilutions for ELISA of pigeon antigens including mucin, TM, PS, PDO, PDF and PIS for ELISA were established by checkerboard analysis (Boyd, 1975; McSharry *et al.*, 2006a).

Initially each antigen was diluted 1/100 in coating buffer (Appendix 2) and 100 µl were placed into the first column of 96 well plates (Maxisorp, Nunc, Denmark). The antigens were double diluted across the plates into coating buffer (figure 3.1) and the plates were incubated overnight at 4° C.

Subsequently, the plates were washed 3 times with PBS-T (Appendix 2) and tap water alternatively and dried on towel paper. Next 100 µl PBS-T-BSA (Appendix 2) was added into each well and the plates were incubated for 1 hour at room temperature. After a further washing and drying step, positive control sera from an asymptomatic fancier with high titre of specific antibodies against pigeon antigens was diluted 1/500 in PBS-T-BSA and 100 µl was placed into the first row of each plate as shown in figure 3.2. The positive control sample was subsequently double diluted into PBS-T-BSA and the plates were incubated at room temperature for 90 minutes.

The plates were washed again a further 3 times, dried and 100 µl of polyclonal rabbit anti-human IgG/HRP (P0214, Sigma, UK) diluted 1/4800 was added to each well and incubated at room temperature for 1 hour.

Finally after a further wash, 100 µl of substrate solution (Appendix 2) was added to each well and the reaction was stopped within 30 minutes at room temperature with 100 µl of 12.5% sulphuric acid (Fisher Scientific, Leicestershire, UK). Absorbance was read in an ELISA reader (Bio-TEK, Vermont, USA) at 490 nm and the results were reported in optical density units (OD).

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											
<b>B</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											
<b>C</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											
<b>D</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											
<b>E</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											
<b>F</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											
<b>G</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											
<b>H</b>	100μl	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800
	1/100	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
	Ag											

**Figure 3.1:** Diagram showing the dilution of pigeon antigen in checker board ELISA.

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control	100 $\mu$ l 1/500 positive control
<b>B</b>	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D	1/1000 D
<b>C</b>	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D	1/2000 D
<b>D</b>	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D	1/4000 D
<b>E</b>	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D	1/8000 D
<b>F</b>	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D	1/16000 D
<b>G</b>	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D	1/32000 D
<b>H</b>	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D	1/64000 D

**Figure 3.2:** Diagram showing the dilution of positive control sera in checker board ELISA;  
D: dilution.

### **3.9: IgG ELISA:**

ELISA assays were based on that described by Baldwin CI et al (Baldwin *et al.*, 1998c).

96 well micro titre plates (Maxisorp, Nunc, Denmark) were coated with 100 µl of pigeon antigens diluted at the optimal dilution as determined by checker board ELISA in coating buffer (Appendix 2), and incubated overnight at 4 °C. The plates were washed 3 times with PBS-T (Appendix 2) and tap water alternatively, dried on towel paper and blocked with 100 µl PBS-T-BSA (Appendix 2) for 1 hour at room temperature.

Serum samples for groups A and B were diluted at 1/500 in PBS-T-BSA and serum samples from group C, D and E at 1/100 (since dilution at 1/500 for group C, D and E had a final results either negative or very low titres).

After a further washing and drying step, 100 µl of diluted test samples were added to the plates and double diluted across the plates into PBS-T-BSA and incubated for 90 minutes at room temperature (figure 3.3). The plates were washed a further time and 100 µl of polyclonal rabbit anti-human IgG/HRP (P0214, Sigma, UK) at 1/4800 was added to each well and incubated at room temperature for 1 hour.

Following 3 further washes 100 µl of substrate solution (Appendix 2) was added into each well and the reaction was stopped within 30 minutes at room temperature with 100 µl of 12.5% sulphuric acid. Absorbance was read in a micro ELISA reader (Bio-TEK, Vermont, USA) at 490 nm.

Absorbance was plotted against the reciprocal of the serum dilution and the dilution that gave an optical density of 0.2 was the antibody titre.



	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	Blank	100 µl 1/500 Positive control	1/1000 Positive control	1/2000 Positive control	1/4000 Positive control	1/8000 Positive control	1/16000 Positive control	1/32000 Positive control	1/64000 Positive control	1/128000 Positive control	1/256000 Positive control	1/512000 Positive control
<b>B</b>	Blank	100 µl 1/500 Sample 1	1/1000 Sample 1	1/2000 Sample 1	1/4000 Sample 1	1/8000 Sample 1	1/16000 Sample 1	1/32000 Sample 1	1/64000 Sample 1	1/128000 Sample 1	1/256000 Sample 1	1/512000 Sample 1
<b>C</b>	Blank	100 µl 1/500 Sample 2	1/1000 Sample 2	1/2000 Sample 2	1/4000 Sample 2	1/8000 Sample 2	1/16000 Sample 2	1/32000 Sample 2	1/64000 Sample 2	1/128000 Sample 2	1/256000 Sample 2	1/512000 Sample 2
<b>D</b>	Blank	100 µl 1/100 Sample 3	1/200 Sample 3	1/400 Sample 3	1/800 Sample 3	1/1600 Sample 3	1/3200 Sample 3	1/6400 Sample 3	1/12800 Sample 3	1/25600 Sample 3	1/51200 Sample 3	1/102400 Sample 3
<b>E</b>	Blank	100 µl 1/100 Sample 4	1/200 Sample 4	1/400 Sample 4	1/800 Sample 4	1/1600 Sample 4	1/3200 Sample 4	1/6400 Sample 4	1/12800 Sample 4	1/25600 Sample 4	1/51200 Sample 4	1/102400 Sample 4
<b>F</b>	Blank	100 µl 1/100 Sample 5	1/200 Sample 5	1/400 Sample 5	1/800 Sample 5	1/1600 Sample 5	1/3200 Sample 5	1/6400 Sample 5	1/12800 Sample 5	1/25600 Sample 5	1/51200 Sample 5	1/102400 Sample 5
<b>G</b>	Blank	100 µl 1/500 Sample 6	1/1000 Sample 6	1/2000 Sample 6	1/4000 Sample 6	1/8000 Sample 6	1/16000 Sample 6	1/32000 Sample 6	1/64000 Sample 6	1/128000 Sample 6	1/256000 Sample 6	1/512000 Sample 6
<b>H</b>	Blank	100 µl 1/500 Sample 7	1/1000 Sample 7	1/2000 Sample 7	1/4000 Sample 7	1/8000 Sample 7	1/16000 Sample 7	1/32000 Sample 7	1/64000 Sample 7	1/128000 Sample 7	1/256000 Sample 7	1/512000 Sample 7

**Figure 3.3:** Diagrammatic set up of ELISA for IgG.

### **3.10: IgG Subclasses ELISA:**

100 µl of pigeon antigen diluted appropriately in coating buffer (Appendix 2) was added to each well of a 96 well plate (Maxisorp, Nunc, Denmark). The plate was incubated at 4° C overnight. The plate was washed 3 times with PBS-T (Appendix 2) and tap water alternatively, dried on towel paper and blocked with 100 µl PBS-T-BSA (Appendix 2) for 1 hour at room temperature.

After a further washing and drying step, 100 µl of appropriately diluted samples in PBS-T-BSA (as described previously in section 3.9) was added to the plate, double diluted in PBS-T-BSA across the plates and incubated overnight at 4° C (figure 3.3).

The plate was washed 3 times and 100 µl monoclonal antihuman IgG1 (clone HP6001, Sigma, UK) or monoclonal antihuman IgG2 (clone HP6002, Sigma, UK) was added to each well for 90 minutes at room temperature.

Subsequently, the plate was washed 3 times and 100 µl of polyclonal rabbit anti-mouse immunoglobulin/HRP (P0260, Dako Cytomation, Denmark) was added to each well and incubated at room temperature for 1 hour. After a further 3 washes 100 µl substrate solution (Appendix 2) was added into each well and the reaction was stopped within 30 minutes at room temperature with 100 µl of 12.5% sulphuric acid. Absorbance was read in an ELISA reader (Bio-TEK, Vermont, USA) at 490 nm.

Absorbance was plotted against the reciprocal of the serum dilution, and the dilution factor that gave an optical density of 0.2 was the antibody titre.

### **3.11: Measurement of Antibody Avidity by Inhibition ELISA:**

100 µl of antigen diluted in coating buffer (Appendix 2) at appropriate concentrations was added to each well of a 96 well plate (Maxisorp, Nunc, Denmark). The plates were incubated overnight at 4° C and washed 3 times in PBS-T (Appendix 2) and tap water alternatively, dried on towel paper and blocked with 100 µl PBS-T-BSA (Appendix 2) for 1 hour at room temperature.

Serum samples for groups A and B were diluted in PBS-T-BSA (Appendix 2) at that dilution that gave an OD of 1 (based on the results of IgG1 and IgG2 ELISA) and 50 µl of each sample was added in every well across the plate.

The chaotropic reagent, ammonium thiocyanate (Sigma, UK) was made up at a range of concentrations (Appendix 3) from 1 to 10 M in PBS and 50 µl of each concentration was added to each sample (figure 3.4). Plates were incubated overnight at 4° C.

Following further washing and drying, 100 µl monoclonal antihuman IgG1 (clone HP6001, Sigma, UK) or monoclonal antihuman IgG2 (clone HP6002, Sigma, UK) was added to each well for 90 minutes at room temperature. Subsequently, the plates were washed 3 times, dried and 100 µl of polyclonal rabbit anti-mouse immunoglobulin/HRP (P0260, Dako cytometry, Denmark) was added to each well and incubated at room temperature for 1 hour.

After a further 3 washes the plates were dried on towel paper and 100 µl substrate (Appendix 2) was added into each well and the reaction was stopped within 30 minutes at room temperature with 100 µl of 12.5% sulphuric acid. Absorbance was read in an ELISA reader (Bio-TEK, Vermont, USA) at 490 nm. The percentage of inhibition (as compared to sera without ammonium thiocyanate) was plotted against the concentration of ammonium thiocyanate and the concentration of ammonium thiocyanate required to reduce the OD by 50% was calculated.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Sample A	sample A+10M AT	Sample A+9 M AT	Sample A+8 M AT	Sample A+7 M AT	Sample A+6 M AT	Sample A+5 M AT	Sample A+4 M AT	Sample A+3 M AT	Sample A+2 M AT	Sample A+1 M AT
B	Blank	Sample A	sample A+10M AT	Sample A+9 M AT	Sample A+8 M AT	Sample A+7 M AT	Sample A+6 M AT	Sample A+5 M AT	Sample A+4 M AT	Sample A+3 M AT	Sample A+2 M AT	Sample A+1 M AT
C	Blank	Sample A	sample A+10M AT	Sample A+9 M AT	Sample A+8 M AT	Sample A+7 M AT	Sample A+6 M AT	Sample A+5 M AT	Sample A+4 M AT	Sample A+3 M AT	Sample A+2 M AT	Sample A+1 M AT
D	Blank	Sample A	sample A+10M AT	Sample A+9 M AT	Sample A+8 M AT	Sample A+7 M AT	Sample A+6 M AT	Sample A+5 M AT	Sample A+4 M AT	Sample A+3 M AT	Sample A+2 M AT	Sample A+1 M AT
E	Blank	Sample B	sample B+10M AT	Sample B+9 M AT	Sample B+8 M AT	Sample B+7 M AT	Sample B+6 M AT	Sample B+5 M AT	Sample B+4 M AT	Sample B+3 M AT	Sample B+2 M AT	Sample B+1 M AT
F	Blank	Sample B	sample B+10M AT	Sample B+9 M AT	Sample B+8 M AT	Sample B+7 M AT	Sample B+6 M AT	Sample B+5 M AT	Sample B+4 M AT	Sample B+3 M AT	Sample B+2 M AT	Sample B+1 M AT
G	Blank	Sample B	sample B+10M AT	Sample B+9 M AT	Sample B+8 M AT	Sample B+7 M AT	Sample B+6 M AT	Sample B+5 M AT	Sample B+4 M AT	Sample B+3 M AT	Sample B+2 M AT	Sample B+1 M AT
H	Blank	Sample B	sample B+10M AT	Sample B+9 M AT	Sample B+8 M AT	Sample B+7 M AT	Sample B+6 M AT	Sample B+5 M AT	Sample B+4 M AT	Sample B+3 M AT	Sample B+2 M AT	Sample B+1 M AT

**Figure 3.4:** Diagram showing the set up of an inhibition ELISA plate; AT: ammonium thiocyanate.

### 3.12: Isothermal Microcalorimeter:

VP-ITC Micro Calorimeter, one of the latest models produced by Microcal (MicroCal LLC, Milton Keynes, UK) (figure 3.5) was used to measure the heat produced in an antigen-antibody reaction (Sigurskjold, Altman & Bundle, 1991). The setting of VP-ITC was as following:

Total injection: 30 times, Cell temperature: 30° C, Reference power: 10  $\mu$ cal/sec, Initial delay: 60 second, Stirring speed: 300, Feedback mode: high, ITC equilibration option: fast, auto, Volume: 5  $\mu$ l, Duration: 10 second, Spacing: 240 second, Filter period: 2 second, Edit mode: all same. Figure 3.6 shows the setting page of VP-ITC.

To begin with, the serum samples from group A and B were diluted 2 times in PBS (Appendix 2) and used as a ligand in the injection syringe. PDO was made at 2 mg/ml in PBS and used as a macro molecule in the sample cell. In order to have a short equilibrium period, the serum sample and PDO were kept on ice. Before running the experiment, the sample and PDO were degassed for 5 minutes with stirring. 1.8 ml pigeon antigen was injected into the reaction cell by the glass syringe. The reference cell was filled with PBS. The injection pipette was placed in the pipette stand into the test tube containing the serum sample and filled with the sample slowly. The tip of the pipette was carefully placed into the reaction cell and the experiment was started.

5  $\mu$ l of the antigen was injected into the reaction cell thirty times with a 240 second gap between each injection to give the reaction cell enough time to return to equilibrium. The heat generated by the antigen-antibody interactions ( $\Delta H$ ) following each injection was measured by the system. A titration curve was obtained when the heat from each injection was plotted against the ratio of antigen and antibody in reaction cell by VP-ITC.  $\Delta H$  was extracted from the curve (Wiseman *et al.*, 1989; Bundle & Sigurskjold, 1994).

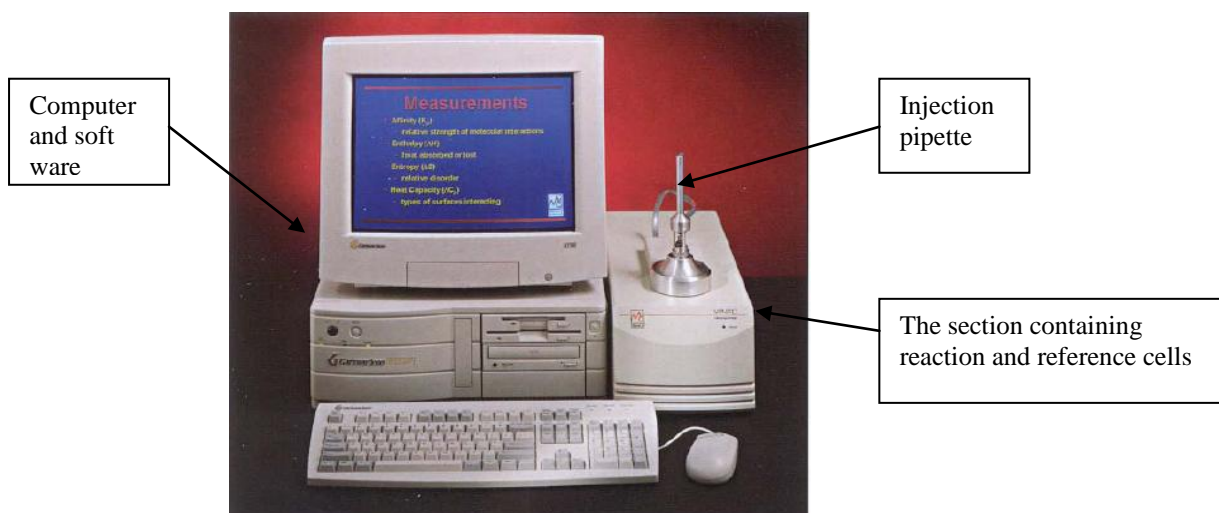


Figure 3.5: VP- ITC Machine.

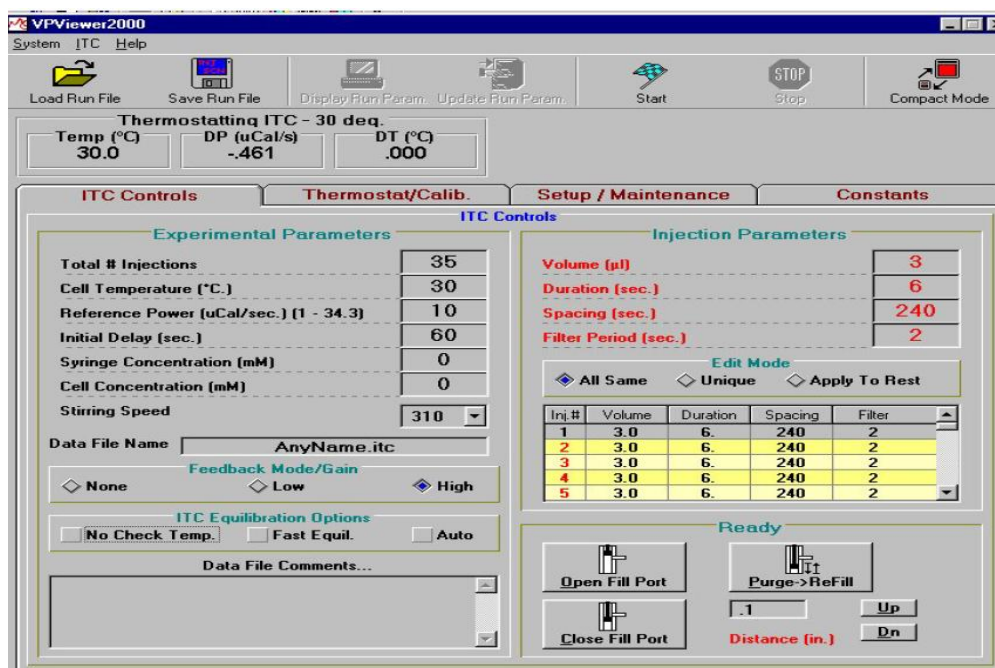
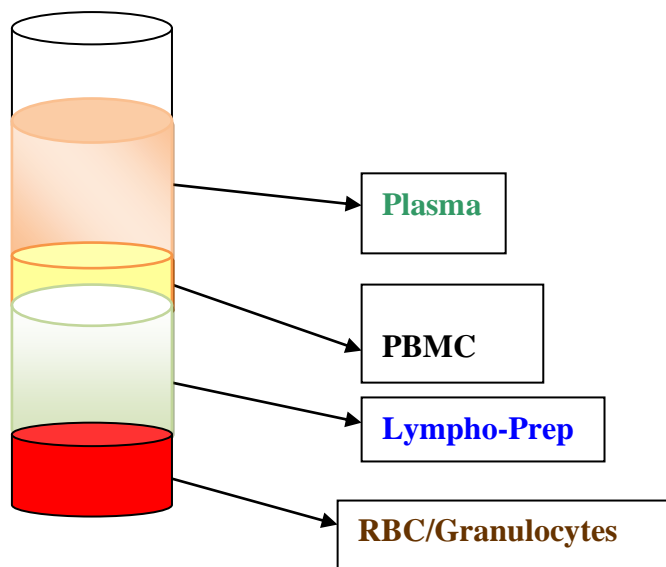


Figure 3.6: VP- ITC setting page before running the experiment.

### 3.13: Isolation of Peripheral Blood Mononuclear Cells:

Freshly heparinised peripheral blood from an asymptomatic fancier was diluted with an equal volume of sterile normal saline. 20 ml of this mixture was carefully layered onto 6 ml Lympho-Prep (Nycomed Pharma As, Norway) in a sterile universal. The preparation was centrifuged at 900 g for 20 minutes at 20° C. The thin layer of peripheral blood mononuclear cells (PBMC) was located between the plasma at the top and Lympho-Prep underneath. Red blood cells and granulocytes centrifuged to the bottom of the tube as shown in figure 3.7.

The PBMC layer was carefully removed by a plastic pipette, washed 2 times in RPMI 1640 (Gibco, Invitrogen, UK) and cells were counted in a haemocytometer as described in section 3.14.



**Figure 3.7:** Visualising the different layers of peripheral blood in the universal bottle.

### 3.14: Counting the Cells by Haemocytometer:

To begin with, the hemocytometer slide was cleaned with 70% alcohol and a cover slip was placed carefully over the grooves and gridded area.

After mixing the cell culture thoroughly to disperse any clumps, the suspension was transferred to the edge of the hemocytometer chamber allowing it to go under the cover slip by capillary action. By using a microscope and tally counter, the cells lying in 25 large squares in the gridded area ( $n$ ) were counted. The cells that situated on the top and left hand lines of each large square were counted to avoid counting cells twice. Since the volume counted was  $0.1 \text{ mm}^3$  or  $1 \times 10^{-4} \text{ ml}$ , the cell concentration in one millilitre ( $c$ ) was calculated from the following formula:

$$c = n \times 10^4$$

### 3.15: Culture Media:

RPMI 1640 (Invitrogen, GIBCO, UK) was used for washing and preparing the cells for culture. RF 10 was used for cell culture and generating EBV transformed B cell lines and it contains:

- inactivated foetal calf serum (FCS) (Invitrogen, UK ): 10% v/v (50 ml)
- L- glutamine (200 mM) (Invitrogen, UK): 1% v/v (5 ml)
- penicillin (100 unit/ml)/streptomycin (100 $\mu$ g/ml) (Invitrogen, UK): 1% v/v (5 ml)
- RPMI 1640 (Invitrogen, Gibco, UK): 500 ml

Prior to use the foetal calf serum was placed in a water bath at  $56^\circ \text{ C}$  for half an hour in order to inactivate complement components which may destroy cells.

RH 10 is another type of fortified media which is similar to RF 10 except it contains inactivated human serum (Invitrogen, UK) instead of inactivated FCS and it was used for T cell cloning.



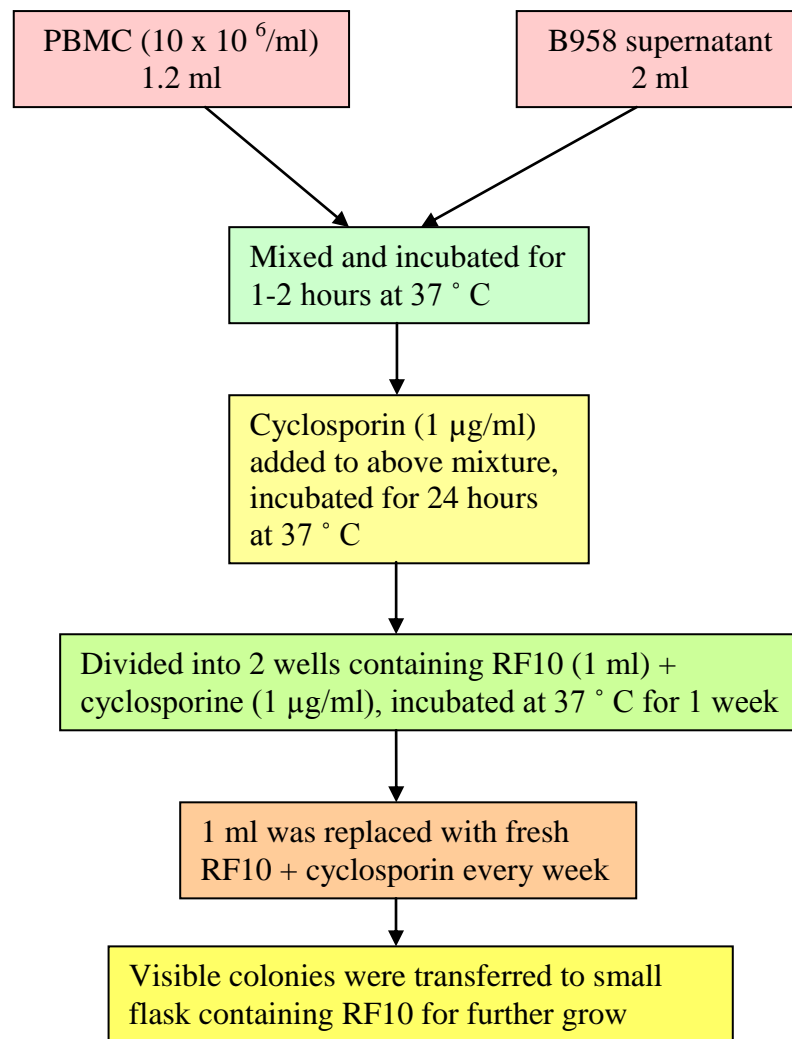
### **3.16: Generation of EBV transformed B cell lines:**

2 different methods were tested. The polymixin B method was faster, more efficient and successful.

#### **3.16.1: EBV/ Cyclosporin Transformed B cell lines:**

PBMC were separated from freshly heparinised peripheral blood from pigeon fanciers.

1.2 ml of PBMC at  $10 \times 10^6$  /ml was incubated with 2 ml supernatant of B958 cells (A kind gift from Dr Desa Lilic, Newcastle University) containing Epstein Barr Virus (EBV) in a humidified condition containing 5% CO<sub>2</sub> at 37° C for 1-2 hours. Cyclosporin (Sandoz, Germany) at a final concentration of 1 µg/ml was added to the mixture, which was incubated for a further 24 hours. This mixture was then transferred into 2 wells in a 12 well culture plate and 1 ml of RF 10/ cyclosporin (1 µg/ml) was added to each well and incubated at 37° C for a week. Cells were monitored and each week 1 ml from each well was replaced with a fresh mixture of RF10 /cyclosporin. After about 3-4 weeks when small colonies of transformed cells were observed, the cells were transferred to small flask (figure 3.8).

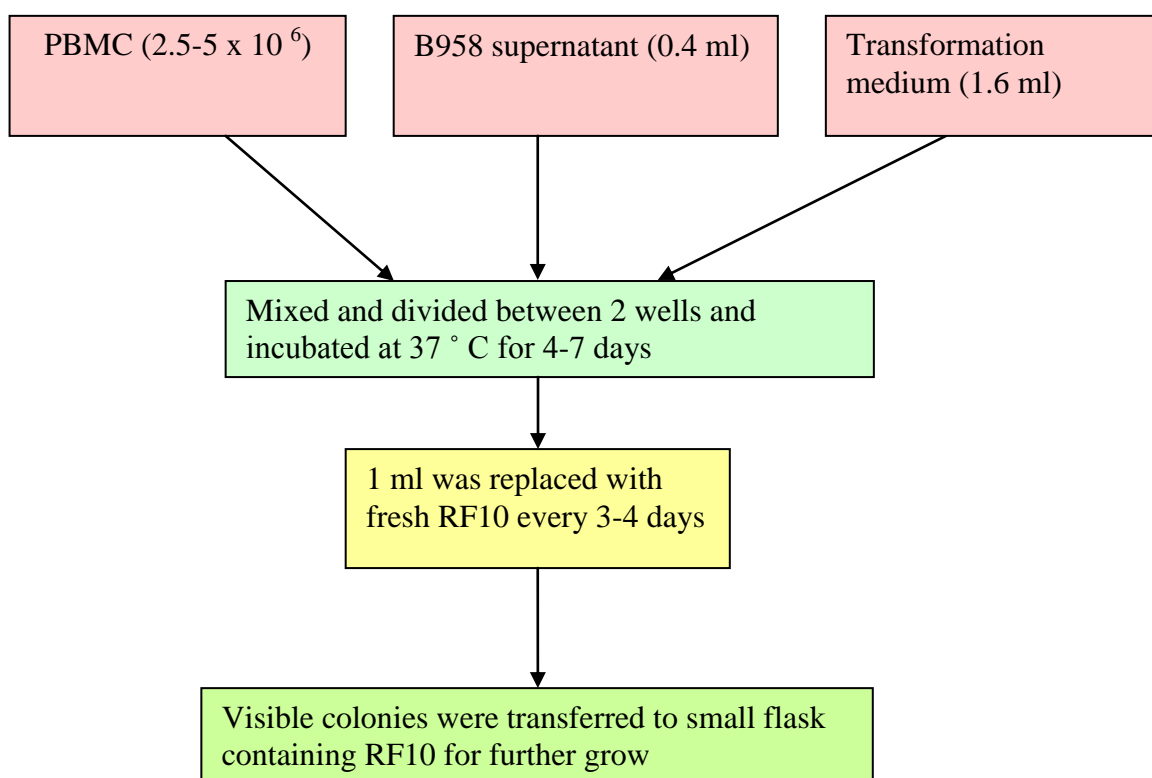


**Figure 3.8:** The flow chart of cyclosporine method for generating of EBV transformed B cell lines.

### **3.16.2: EBV/ Polymyxin B Transformed B cells:**

PBMC were separated from freshly heparinised peripheral blood from pigeon fanciers.

$2.5 - 5 \times 10^6$  PBMC were placed in 1.6 ml of transformation medium [containing 5 ml RPMI, 20% v/v inactivated FCS, 1% v/v 200 mM L-glutamine, 1% v/v phytohaemagglutinin (PHA, Sigma, UK), 100 units/ml penicillin/streptomycin and 100 units/ml polymyxin B-sulphate (Sigma, UK)] and the EBV supernatant of B958 cell culture in the ratio of 4:1 (0.4 ml) ((Bass, Walters & Darke, 2004). 1 ml of that mixture was placed into 2 wells of the 24-well, flat-bottomed culture plate and incubated in humidified condition containing 5% CO<sub>2</sub> at 37° C for 4-7 days until the visible B cell line was established. Every 3-4 days 1 ml from each well was replaced with a fresh mixture of RF 10. After about 3-4 weeks the cells were transferred to small flask (figure 3.9).



**Figure 3.9:** The flow chart of polymyxin B method for generating of EBV transformed B cell lines.

### **3.17: Irradiation of the Feeder Cells:**

To produce growth arrested feeder cell lines, the EBV transformed B cells were irradiated by “Gamma Cell 1000 Elite” (MDS Nordion, Ottawa, Canada) at 60 Gray ((Puck & Marcus, 1956). At this dosage of Gamma radiation, the cells survive but stop dividing (Dr Baldwin personal communication).

### **3.18: T cell cloning:**

The method choice for T cells cloning is dilution cloning in which a cell suspension is diluted to the point that a chance of having more than one cell in the inoculum's volume is negligible (Newman, 1996).

The process of cloning is shown in figure 3.10. Peripheral blood lymphocytes were made up at  $5 \times 10^5$  /ml and seeded within 2 sets of 96 well cell culture plates as follow:

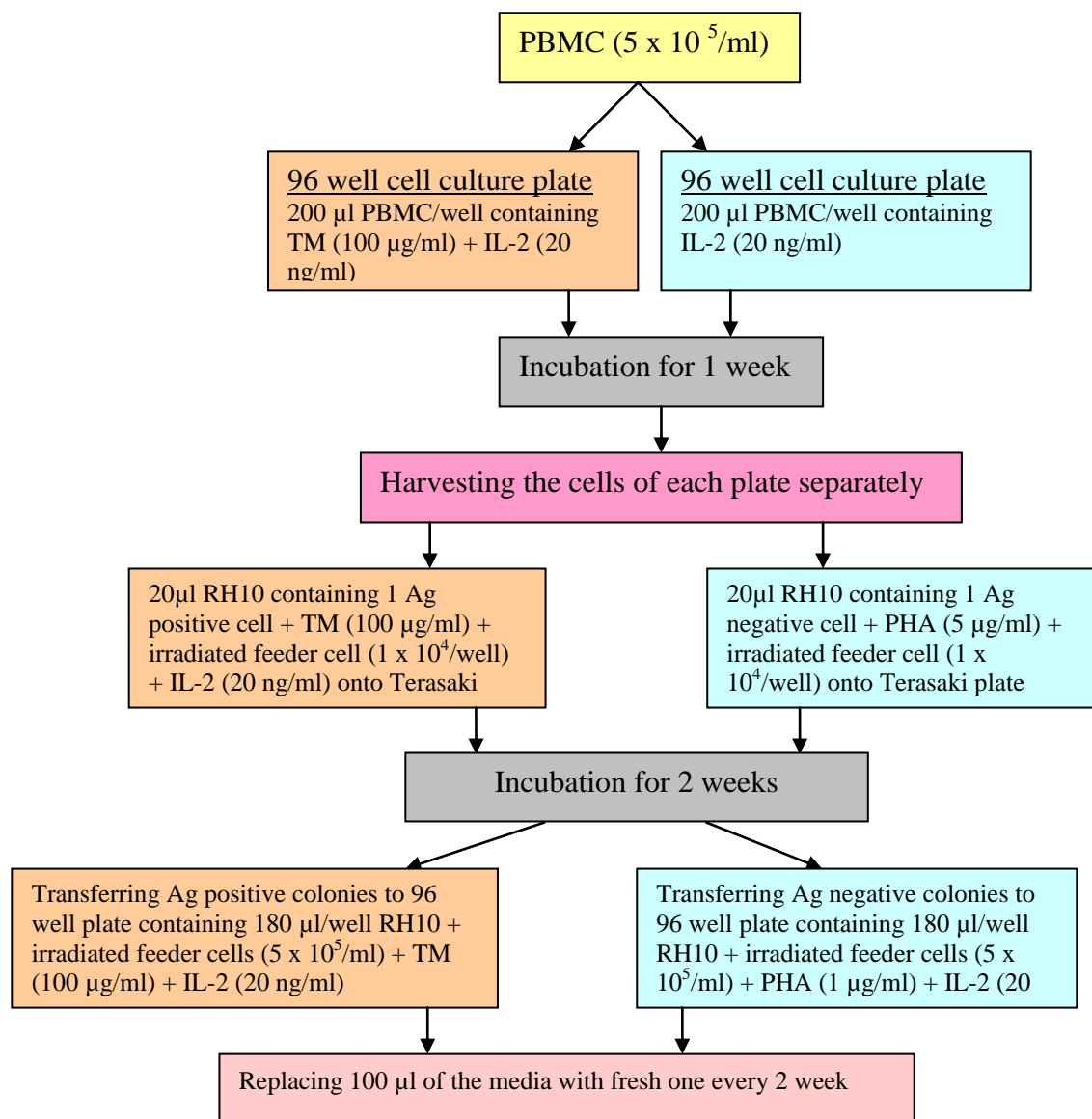
- cells with antigen ( Ag positive cells): lymphocytes + t mucin (100 µg/ml) + IL-2 (20 ng/ml)
- cells without antigen ( Ag negative cells): lymphocytes + IL-2 (20 ng/ml)

Plates were incubated in a humidified incubator containing 5% CO<sub>2</sub> at 37° C for one week. The cells were harvested into 2 separate tubes, washed three times with RPMI 1640, counted on a haemocytometer and diluted. 20 µl of media containing Ag positive cells (1 cell/20µl), t mucin (100 µg/ml), irradiated feeder cells ( $1 \times 10^4$  /well) and IL-2 (20 ng/ml) was aliquated into Terasaki plates (GBO, UK) (Figure 3.11).

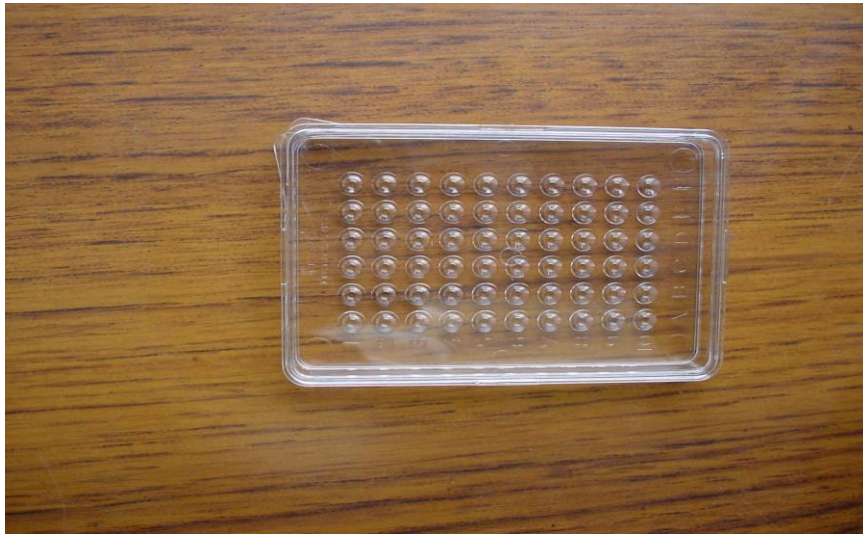
Also 20  $\mu$ l of media containing Ag negative cells (1 cell/ 20 $\mu$ l), irradiated feeder cells ( $1 \times 10^4$  /well) and PHA (5  $\mu$ g/ml, Sigma, UK) was seeded onto a separate Terasaki plate. The plates were incubated in a humidified chamber at 37° C in 5% CO<sub>2</sub> for 2 weeks.

After 2 weeks, colonies were selected and transferred into two 96 well round bottom culture plates. Subsequently, 180  $\mu$ l of irradiated feeder cells ( $5 \times 10^5$  /ml), t mucin (100  $\mu$ g/ml), and IL-2 (20 ng/ml) were added to the wells containing Ag positive cells or PHA (1  $\mu$ g/ml), IL-2 and irradiated feeder cells to the wells containing Ag negative cells. Every 2 weeks 100  $\mu$ l of the media was replaced with the fresh mixtures.

The cells were monitored regularly and when visible cell colonies were established, the cells were transferred into 2 wells. Cells were grown up into larger volumes when appropriate and after 4-6 months the clones were stored in liquid nitrogen (LN2) for further experiments.



**Figure 3.10:** The flow chart of T cell cloning.



**Figure 3.11:** Terasaki plate; each well has 20  $\mu$ l volume.



### **3.19: Freezing down the Cells in Liquid Nitrogen:**

Cells were harvested and washed twice with RPMI 1640 and then made up to  $1-2 \times 10^6$  /ml in inactivated FCS containing 10% Dimethyl Sulfoxide (DMSO, Pharmingen, UK).

Cells were aliquoted into the cryovials (Sigma, UK) and then placed at  $-80^\circ \text{C}$  in a polystyrene container for 24 hours and subsequently transferred into liquid nitrogen.

### **3.20: Proliferation Assay:**

Cell proliferation was determined by the use of radiolabeled DNA precursors, in this case tritiated thymidine ( $^3\text{H}$ -thymidine) (Merion, Howell & Bromberg, 1998; Laughlin *et al.*, 2007). The amount of radiation detected in the cells is proportional to proliferative response of the cells (Merion, Howell & Bromberg, 1998; Laughlin *et al.*, 2007).

T cell clones and irradiated feeder cells were made up at  $2.5 \times 10^5$  /ml and 200  $\mu\text{l}$  of this was added into the wells of a 96 well plate as shown in figure 3.12. Cells were incubated with IL-2 (20 ng/ml), PHA or t mucin (at 200, 50, and 10  $\mu\text{g/ml}$ ) respectively in triplicate at  $37^\circ \text{C}$  for 4 days. On the 4th day of incubation, 0.0185 MBq  $^3\text{H}$ -thymidine was added to each well. After 6 hours incubation, the T cell clones were harvested into a glass fibre filter by Packard Filter mate Harvester (Packard Instrument Company, Meriden, CT, USA) (Figure 3.13). The next day the filters were processed for counting in a flat beta plate counter (Packard Instrument Company, Meriden, CT, USA). Following calculation of mean values of each triplicate, the stimulation index (SI) which is the ratio of the counts per minute of radioactivity in cells stimulated by t mucin, IL-2 or PHA divided by the counts per minute for unstimulated cells was determined.

1	2	3	4	5	6	7	8	9	10	11	12
RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI
RPMI	clone 01+ feeders+ media	Clone 01+ feeders + TM (200µg/ml)	Clone 01+ feeders + TM (50µg/ml)	Clone 01+ feeders + TM (10µg/ml)	Clone 01+ feeders + PHA (10µg/ml)	Clone 01+ feeders + IL2 (20ng/ml)					RPMI
RPMI	Clone 01+ feeders + media	Clone 01+ feeders + TM (200µg/ml)	Clone 01+ feeders + TM (50µg/ml)	Clone 01+ feeders + TM (10µg/ml)	Clone 01+ feeders + PHA (10µg/ml)	Clone 01+ feeders + IL2 (20ng/ml)					RPMI
RPMI	Clone 01+ feeders + media	Clone 01+ feeders + TM (200µg/ml)	Clone 01+ feeders + TM (50µg/ml)	Clone 01+ feeders + TM (10µg/ml)	Clone 01+ feeders + PHA (10µg/ml)	Clone 01+ feeders + IL2 (20ng/ml)					RPMI
RPMI	Clone 02 + feeders+ media	Clone 02+ feeders + TM (200µg/ml)	Clone 02+ feeders + TM (50µg/ml)	Clone 02+ feeders + TM (10µg/ml)	Clone 02+ feeders + PHA (10µg/ml)	Clone 02+ feeders + IL2 (20ng/ml)					RPMI
RPMI	Clone 02 + feeders+ media	Clone 02+ feeders + TM (200µg/ml)	Clone 02+ feeders + TM (50µg/ml)	Clone 02+ feeders + TM (10µg/ml)	Clone 02+ feeders + PHA (10µg/ml)	Clone 02+ feeders + IL2 (20ng/ml)					RPMI
RPMI	Clone 02 + feeders+ media	Clone 02+ feeders + TM (200µg/ml)	Clone 02+ feeders + TM (50µg/ml)	Clone 02+ feeders + TM (10µg/ml)	Clone 02+ feeders + PHA (10µg/ml)	Clone 02+ feeders + IL2 (20ng/ml)					RPMI
RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI

**Figure 3.12:** Diagram of preparation of the plate for proliferation assay.



**Figure 3.13:** Packard Filter mate Harvester

### **3.21: Cytokine Profile:**

$2 \times 10^5$  T cell clones were incubated with and without the stimulators; Ionomycin (2  $\mu\text{g/ml}$ , Sigma, UK) and Phorbol 12 Myristate 13- Acetate (PMA, 10  $\text{ng/ml}$ , Sigma, UK); in triplicate at  $37^\circ\text{C}$  for one to five days as shown in figure 3.14. Each day the supernatant of the clones were collected and stored at  $-20^\circ\text{C}$ . Finally the supernatant was analysed for different cytokines by ELISA.

1	2	3	4	5	6	7	8	9	10	11	12
	clone 04	clone 05	feeder cells								
RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI
RPMI	2x 10 <sup>5</sup> cells, no stimulator	2x 10 <sup>5</sup> cells, no stimulator	2x 10 <sup>5</sup> cells, no stimulator								RPMI
RPMI	2x 10 <sup>5</sup> cells, no stimulator	2x 10 <sup>5</sup> cells, no stimulator	2x 10 <sup>5</sup> cells, no stimulator								RPMI
RPMI	2x 10 <sup>5</sup> cells, no stimulator	2x 10 <sup>5</sup> cells, no stimulator	2x 10 <sup>5</sup> cells, no stimulator								RPMI
RPMI	2x 10 <sup>5</sup> cells + stimulator	2x 10 <sup>5</sup> cells + stimulator	2x 10 <sup>5</sup> cells + stimulator								RPMI
RPMI	2x 10 <sup>5</sup> cells + stimulator	2x 10 <sup>5</sup> cells + stimulator	2x 10 <sup>5</sup> cells + stimulator								RPMI
RPMI	2x 10 <sup>5</sup> cells + stimulator	2x 10 <sup>5</sup> cells + stimulator	2x 10 <sup>5</sup> cells + stimulator								RPMI
RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI	RPMI

**Figure 3.14:** Diagram of preparing the plate for cytokine profile.

### **3.22: Cytokine ELISA:**

The supernatants collected after stimulation with PMA and Ionomycin were tested for different cytokines (Appendix 5) by ELISA.

96 well plates (Maxisorp, Nunc, Denmark) were coated with 100 µl of specific anti-cytokine antibody (Pharminogen, BD Bioscience, UK) diluted appropriately (Appendix 4) in the coating buffer (Appendix 2) and incubated in a humidified chamber at 4° C overnight. The following day the content of the wells was discarded and the plate was dried on tissue paper. 100 µl PBS-T-BSA (Appendix 2) was added into the wells, incubated at room temperature for one hour. Subsequently the plate was washed 3 times in PBS-T (Appendix 2) and dried.

The top standard (NIBSC, Hertfordshire, UK) (concentration of the top standards are shown in section Appendix 5) was double diluted down in the plate in PBS-T-BSA (Appendix 2) and 100 µl of ½ diluted supernatant in PBS-T-BSA (Appendix 2) was added to the rest of the wells and the plate was incubated at 37° C for one hour (figure 3.15). Following a further 3 washes, the plate was dried and 100 µl of diluted biotinylated anti-cytokine antibody (Pharminogen, BD Bioscience, UK) in PBS-T-BSA (dilution of each biotinylated antibody are shown in Appendix 6) was added to each well and the plate was incubated at 37° C for one hour.

Subsequently after a further washing and drying step, 100 µl of substrate solution (Appendix 2) was added to each well and the plate was incubated for 30 minutes in the dark. Finally 100 µl of 12.5% sulphuric acid was added to each well to stop the reaction and OD was read by using an ELISA Ultra Microplate Reader (Bio-TEK, Vermont, USA) at 490 nm. The standard curve was determined by plotting the concentrations against the OD of the standard.

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	Top standard	Top standard	½ clone 01(S <sup>-</sup> ) D1	½ clone 01(S <sup>+</sup> ) D1	½ clone 01(S <sup>-</sup> ) D2	½ clone 01(S <sup>+</sup> ) D2	½ clone 01(S <sup>-</sup> ) D3	½ clone 01(S <sup>+</sup> ) D3	½ clone 01(S <sup>-</sup> ) D4	½ clone 01(S <sup>+</sup> ) D4	½ clone 01(S <sup>-</sup> ) D5	½ clone 01(S <sup>+</sup> ) D5
<b>B</b>	½ top standard	½ top standard	½ clone 02(S <sup>-</sup> ) D1	½ clone 02(S <sup>+</sup> ) D1	½ clone 02(S <sup>-</sup> ) D2	½ clone 02(S <sup>+</sup> ) D2	½ clone 02(S <sup>-</sup> ) D3	½ clone 02(S <sup>+</sup> ) D3	½ clone 02(S <sup>-</sup> ) D4	½ clone 02(S <sup>+</sup> ) D4	½ clone 02(S <sup>-</sup> ) D5	½ clone 02(S <sup>+</sup> ) D5
<b>C</b>	¼ top standard	¼ top standard	½ clone 03(S <sup>-</sup> ) D1	½ clone 03(S <sup>+</sup> ) D1	½ clone 03(S <sup>-</sup> ) D2	½ clone 03(S <sup>+</sup> ) D2	½ clone 03(S <sup>-</sup> ) D3	½ clone 03(S <sup>+</sup> ) D3	½ clone 03(S <sup>-</sup> ) D4	½ clone 03(S <sup>+</sup> ) D4	½ clone 03(S <sup>-</sup> ) D5	½ clone 03(S <sup>+</sup> ) D5
<b>D</b>	1/8 top standard	1/8 top standard	½ clone 04(S <sup>-</sup> ) D1	½ clone 04(S <sup>+</sup> ) D1	½ clone 04(S <sup>-</sup> ) D2	½ clone 04(S <sup>+</sup> ) D2	½ clone 04(S <sup>-</sup> ) D3	½ clone 04(S <sup>+</sup> ) D3	½ clone 04(S <sup>-</sup> ) D4	½ clone 04(S <sup>+</sup> ) D4	½ clone 04(S <sup>-</sup> ) D5	½ clone 04(S <sup>+</sup> ) D5
<b>E</b>	1/16 top standard	1/16 top standard	½ clone 05(S <sup>-</sup> ) D1	½ clone 05(S <sup>+</sup> ) D1	½ clone 05(S <sup>-</sup> ) D2	½ clone 05(S <sup>+</sup> ) D2	½ clone 05(S <sup>-</sup> ) D3	½ clone 05(S <sup>+</sup> ) D3	½ clone 05(S <sup>-</sup> ) D4	½ clone 05(S <sup>+</sup> ) D4	½ clone 05(S <sup>-</sup> ) D5	½ clone 05(S <sup>+</sup> ) D5
<b>F</b>	1/32 top standard	1/32 top standard	½ clone 06(S <sup>-</sup> ) D1	½ clone 06(S <sup>+</sup> ) D1	½ clone 06(S <sup>-</sup> ) D2	½ clone 06(S <sup>+</sup> ) D2	½ clone 06(S <sup>-</sup> ) D3	½ clone 06(S <sup>+</sup> ) D3	½ clone 06(S <sup>-</sup> ) D4	½ clone 06(S <sup>+</sup> ) D4	½ clone 06(S <sup>-</sup> ) D5	½ clone 06(S <sup>+</sup> ) D5
<b>G</b>	1/64 top standard	1/64 top standard	½ clone 07(S <sup>-</sup> ) D1	½ clone 07(S <sup>+</sup> ) D1	½ clone 07(S <sup>-</sup> ) D2	½ clone 07(S <sup>+</sup> ) D2	½ clone 07(S <sup>-</sup> ) D3	½ clone 07(S <sup>+</sup> ) D3	½ clone 07(S <sup>-</sup> ) D4	½ clone 07(S <sup>+</sup> ) D4	½ clone 07(S <sup>-</sup> ) D5	½ clone 07(S <sup>+</sup> ) D5
<b>H</b>	1/128 top standard	1/128 top standard	½ feeder (S <sup>-</sup> ) D1	½ feeder (S <sup>+</sup> ) D1	½ feeder (S <sup>-</sup> ) D2	½ feeder (S <sup>+</sup> ) D2	½ feeder (S <sup>-</sup> ) D3	½ feeder (S <sup>+</sup> ) D3	½ feeder (S <sup>-</sup> ) D4	½ feeder (S <sup>+</sup> ) D4	½ feeder (S <sup>-</sup> ) D5	½ feeder (S <sup>+</sup> ) D5

**Figure 3.15:** Diagram showing the set up of cytokine ELISA; (S<sup>+</sup>): supernatants with stimulator, (S<sup>-</sup>): supernatant without stimulator, D: day.

### **3.23: Fluorescence- Activated Cell Sorting (FACs) Analysis:**

Each T cell clone was stained with a range of specific antibodies (Bioscience, UK) to determine the phenotype of the cells. These antibodies were attached to T lymphocytes as a single color direct immunofluorescence reagent detected when the flow cytometer light excited the fluorochrome.

For each clone 2 tubes were prepared. 5 µl anti- CD3 (FITC, Fluorescein Isothiocyanate, producing green fluorescence), 5 µl of anti- CD4 (PE, Phycoerythrin) were added to the first test tube. The second test tube contained 5 µl anti- CD3 (FITC), 5 µl of anti- CD8 (PE). Subsequently  $2.5 \times 10^5$  of T cell clone were added to each tube and the total volume was made up to 300 µl with RPMI 1640. Tubes were incubated in a dark place at room temperature for 20 minutes and finally the tubes were analyzed by a Becton Dickinson FACs machine (Becton Dickinson Company, San Diego, USA).

### **3.24: Safety Statement:**

Pigeon antigens were considered to be class 2 substances and were handled appropriately.

Human products were also handled as following class 2 health and safety guidelines.

Tritiated thymidine or  $^3\text{H}$ -thymidine emits  $\beta$  particle and was handled as prescribed by the Northumbria University Radiation Safety code. The operator attended in the radiation safety training course (Northumbria University, 2003).



### **3.25: Statistical Analysis:**

Statistical analysis was performed using Mann Whitney U test, multiple regression and Chi-square where the data was nonparametric and student t test where the data was parametric. All statistical calculation was carried out on SPSS version 15. A P value  $< 0.05$  was considered significant.

## **4: The Role of Serum Antibodies in Pigeon Fanciers' Lung**

### **4.1: Introduction:**

#### **4.1.1: Antibody:**

##### **4.1.1.1: The Structure of Antibodies:**

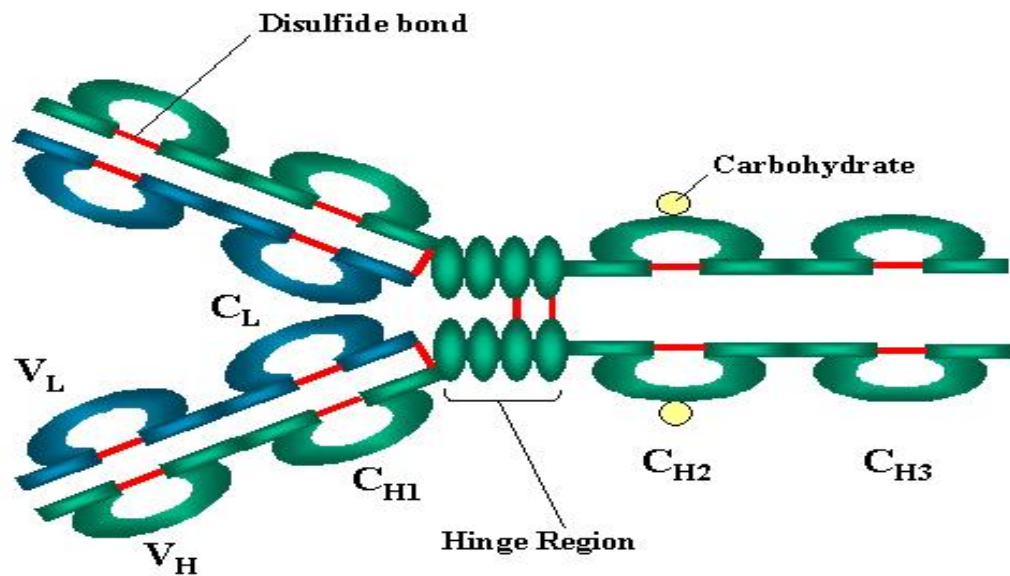
The humoral immune response plays an effective role in protection against foreign antigens (Boes *et al.*, 1998; Rogers *et al.*, 2006). Antibodies are produced by B cells and exist as either membrane bound immunoglobulin which interacts with antigen, or secreted antibodies.

A schematic diagram of an IgG molecule is presented (Figure 4.1) as this molecule demonstrates the shared basic structure of all antibodies. The IgG molecule contains four different polypeptide chains: 2 identical heavy (H) and 2 identical light (L) polypeptide chains. The chains are linked together by interchain disulphydryl bonds (S-S) between cysteine residues. The light chains are 2 identical kappa ( $\kappa$ ) or lambda ( $\lambda$ ) and the heavy chains are alpha ( $\alpha$ ), gamma ( $\gamma$ ), delta ( $\delta$ ), epsilon ( $\epsilon$ ), or mu ( $\mu$ ) (Clark, 1997). The amino terminal domains of both the H and L chains have different amino acid sequences which form the variable regions ( $V_H$ ,  $V_L$ ) or domains. These variable domains allow antibodies to recognise a huge diversity of antigens. The  $\gamma$  heavy chain has 3 domains ( $C_{H1}$ ,  $C_{H2}$ , and  $C_{H3}$ ) whilst the light chain has one constant domain. The constant domain of the H chains has limited variability and determines the biological functions of each isotype.

The hypervariable regions within the  $V_H$  and  $V_L$  domains show a high level of amino acid sequence variability between different antibodies whilst the framework regions show less variability. A cleft formed by a folded  $V_H$  and  $V_L$  domains brings the hypervariable regions together and this is the antigen-binding site. The complementary determining regions of antibodies (CDR) are the sites of closest contact between antigen and antibody. The  $C_{H2}$  domain of FC section interacts with the complement cascade, and the  $C_{H2}$  and  $C_{H3}$

domains bind phagocyte via FC receptors (FCRs) to stimulate the antigen uptake (Michaelson, Garred & Aase, 1991).

Antibodies are classified as different isotypes based on their heavy chain. There are structural variations in the quaternary structure and the hinge length between isotypes. There are different quaternary arrangement including monomers such as IgG, IgD and IgE, dimmers such as secretory IgA and pentamers such as IgM. This structure determines the isotype size and therefore its distribution in the body. IgG can be further divided into the subclasses IgG1, IgG2, IgG3 and IgG4 and IgA can be divided into IgA1 and IgA2. Biological preparations of the immunoglobulin are shown in Table 4.1.



**Figure 4.1:** Schematic diagram of an IgG molecule. It has 2 identical heavy (H) and 2 identical light (L) chains. Each heavy chain has one variable domain (V<sub>H</sub>) and 3 constant domains (C<sub>H</sub>1, C<sub>H</sub>2 and C<sub>H</sub>3). Each light chain has one variable (V<sub>L</sub>) and one constant (C<sub>L</sub>) domain. Antigen binding fragment (Fab) contains one entire L chain and about half of H chain. The other half of H chain is crystalizable fragment (Fc) which contains FC receptor-binding sites (Mayer, 2008).

Isotype	Molecular Weight (kDa)	% of total Ig (adult serum)	Biological half-life (days)	Trans-placental transfer	Complement activation via classical pathway	Biological properties
<b>IgA<sub>1</sub></b>	160-300	11-14	5.9	-	-	Toxin neutralizing, agglutinating, bacteriolytic
<b>IgA<sub>2</sub></b>	160-350	1-4	4.5	-	-	Toxin neutralizing, agglutinating, bacteriolytic
<b>IgD</b>	175	0.2	2-8	-	-	Mature B cell marker
<b>IgE</b>	190	0.004	1-5	-	-	Mediate changes in vascular permeability
<b>IgG<sub>1</sub></b>	150	45-53	21-24	+	+++	Toxin neutralizing, opsonising, agglutinating, bacteriolytic
<b>IgG<sub>2</sub></b>	150	11-15	21-24	+	+	Toxin neutralizing, opsonising, agglutinating, bacteriolytic
<b>IgG<sub>3</sub></b>	160	3-6	7-8	+	++++	Toxin neutralizing, opsonising, agglutinating, bacteriolytic
<b>IgG<sub>4</sub></b>	150	1-4	21-24	+	-	Toxin neutralizing, opsonising, agglutinating, bacteriolytic
<b>IgM</b>	950	10	5-10	-	++++	Primary antibody response, toxin neutralizing, agglutinating, bacteriolytic,

**Table 4.1:** The details about the half-life of different antibodies, the longest half-life belongs to IgG subclasses (Paul, 2003).

#### 4.1.1.2: IgG Fc Receptors:

Receptors for immunoglobulin interact with the FC domains of antibodies and link humoral and cellular aspects of the immune responses (Salmon & Pricop, 2001). IgG Fc receptors are termed Fc $\gamma$ R which exist on macrophages, monocytes, neutrophils, eosinophils, NK cells and B cells (Table 4.2) (Sarfati *et al.*, 1992; Bazilio *et al.*, 2004; Selvaraj *et al.*, 2004). They are encoded by members of the immunoglobulin superfamily of genes which are present on the long arm of chromosome 1 (1q21–23) (Salmon & Pricop, 2001).

Three main subclasses of IgG Fc receptors have been identified with FC $\gamma$ RI (CD64) having a high affinity for human IgG1 and IgG3 (Akerley, Guyre & Davis, 1991) and FC $\gamma$ RII (CD32) and FC $\gamma$ RIII having a low affinity for IgG (Hogg, 1988; Bazilio *et al.*, 2004). FC $\gamma$ RII has been classified into 3 isoforms of A, B and C based on their cell distribution and stimulatory or inhibitory functions which depends on the presence of immunoreceptor tyrosine-based activation/inhibition motif (ITAM/ITIM) in their intracytoplasmic domain (Salmon & Pricop, 2001; Bazilio *et al.*, 2004).

Stimulatory FC $\gamma$ R initiates phagocytosis, antibody-dependent cell mediated cytotoxicity (ADCC) and the release of cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-6 and IL-12 (Bazilio *et al.*, 2004). These receptors have an important role in type II and type III hypersensitivity reactions. On the other hand, inhibitory FC $\gamma$ R (Fc $\gamma$ RIIb) can abolish cellular signalling and play a central role as negative regulators of immune complex-triggered activation (Wasowska *et al.*, 2007).

Following interaction of stimulatory FC $\gamma$ R with its ligand (Fc region), FC $\gamma$ R are clustered and cross-linked at the cell surface by multivalent antigen-antibody complexes which induce phosphorylation of ITAM by tyrosine kinases (Salmon & Pricop, 2001; Bazilio *et al.*, 2004). As a consequence, SyK kinase is recruited which activates phospholipase C and

therefore production of phosphoinositol messengers and increases cytoplasmic calcium. This signalling pathway triggered by FC $\gamma$ R reaches the nucleus leading to activation of transcription factors (Salmon & Pricop, 2001; Pan & Pei, 2003; Swanson & Hoppe, 2004). On the other hand, interaction of inhibitory FC $\gamma$ R with FC domain can lead to the phosphorylation of ITIM, recruitment of SH2-containing inositol polyphosphate 59-phosphatase (SHIP) and prevention of calcium influx (Salmon & Pricop, 2001).

One of the host defence mechanisms triggered by the humoral immune response is ADCC, a lytic attack on antibody tagged cells. This triggers the release of cytokines such as TNF- $\alpha$  and IFN- $\gamma$ , and cytotoxic granules containing perforin (cytolytic protein) and granzymes (serine protease) from NK cells resulting in elimination of antibody covered target cells (Sun, 2003; Niwa *et al.*, 2005). The structure of the oligosaccharides linked to the CH2 domain of the FC region of antibody may also influence ADCC. It has been reported that removal of fucose from IgG1 oligosaccharides can enhance ADCC activity *in vitro* (Niwa *et al.*, 2005).

It has been suggested that FC $\gamma$ R polymorphisms may play an important role in an individual's susceptibility to immune complex-mediated diseases (Zuniga *et al.*, 2003; Bazilio *et al.*, 2004). For instance, FC $\gamma$ RIIa has 2 expressed alleles, R131 and H131 (arginine or histidine at position 131, respectively), in the extracellular domain of the molecule (Bazilio *et al.*, 2004). FC $\gamma$ RIIa-H131 binds to IgG2 efficiently and has been shown to have an essential role in the handling and clearance of IgG2 immune complexes (Bazilio *et al.*, 2004). Polymorphisms in FC $\gamma$ RIIa have been associated with the development of a number of diseases such as SLE (Yuan *et al.*, 2008), severe *Falciparum* malaria (Sinha *et al.*, 2008) and invasive *Streptococcus pneumonia* infection (Yuan *et al.*, 2008).

Receptor name	Principal antibody ligand	Affinity for ligand	Cell distribution	Effect following binding to antibody
FcγRI (CD64)	IgG1 and IgG3	High	Macrophages Neutrophils Eosinophils Dendritic cells	Phagocytosis Cell activation Activation of respiratory burst Induction of microbe killing
FcγRIIA (CD32)	IgG	Low	Macrophages Neutrophils Eosinophils Platelets Langerhans cells	Phagocytosis Degranulation (eosinophils)
FcγRIIB1 (CD32)	IgG	Low	B Cells Mast cells	No phagocytosis Inhibition of cell activity
FcγRIIB2 (CD32)	IgG	Low	Macrophages Neutrophils Eosinophils	Phagocytosis Inhibition of cell activity
FcγRIIA (CD16a)	IgG	Low	NK cells	Induction of antibody-dependent cell-mediated cytotoxicity (ADCC)
FcγRIIB (CD16b)	IgG	Low	Eosinophils Macrophages Neutrophils Mast cells Follicular dendritic cells	Induction of microbe killing

**Table 4.2:** IgG FC receptors and their antibody ligands, affinity, cell distributions and functions (Fridman, 1991; Indik *et al.*, 1995).



#### **4.1.1.3: Antibody Isotype Switching:**

Following genomic rearrangement of the immunoglobulin gene using V(D)J recombination, membrane IgM and IgD molecules are expressed on naive mature B cells (Maizels, 2005). These membrane expressed antibodies are critical in primary antibody production and their expressions are independent of both antigen and T cells. Three lymphoid specific proteins are involved in this rearrangement are recombination activation genes (RAG1, RAG2) and terminal deoxynucleotidyl transferase (Neuberger *et al.*, 2000). The first 2 are involved in recognition and cutting of rearrangement signal sequences and the third is responsible in adding nucleotides to the junctions of V, D and J segments (Neuberger *et al.*, 2000).

The secondary antibody repertoire is shaped in the lymph nodes, mainly in germinal centres, tonsils and spleen and is an antigen and T cell dependent process which involves class-switch recombination (CSR) and somatic hypermutation (SHM) in the immunoglobulin gene (Imai *et al.*, 2003). Both require successful transcription of target DNA sequences, DNA cleavage, and DNA repair (Notarangelo *et al.*, 2006). CSR is a DNA recombination event in the immunoglobulin gene which replaces the C $\gamma$  exon with different C $\gamma$ , C $\alpha$ , or C $\epsilon$  resulting in the production of different isotypes (Bransteitter *et al.*, 2006). The V region and therefore the antibody specificity and affinity stay intact (Zarrin *et al.*, 2005).

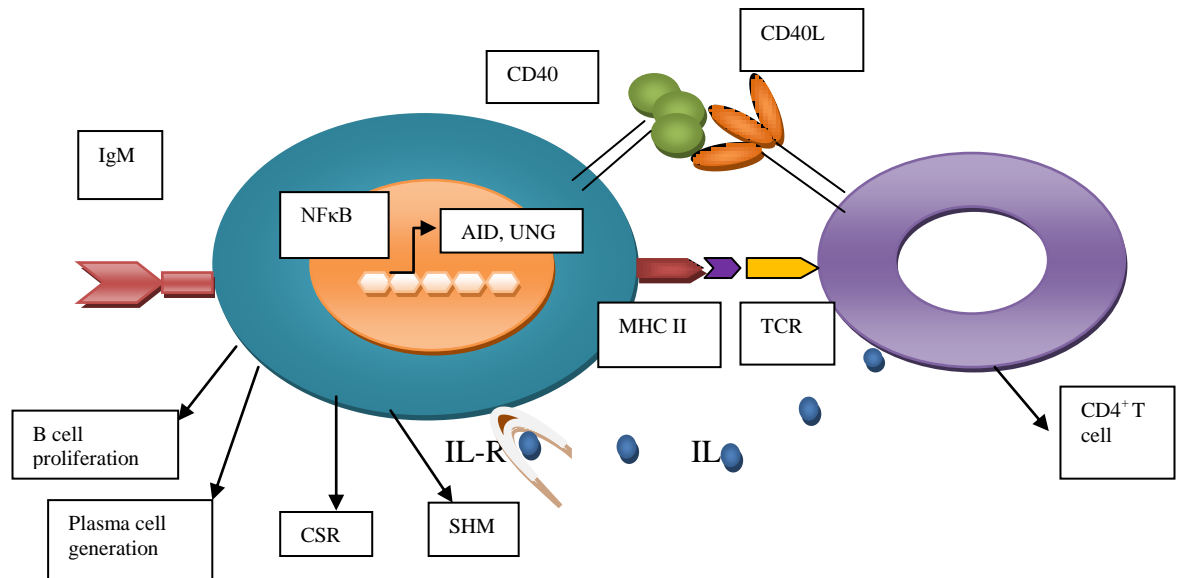
On the other hand, SHM generates high frequency mutations downstream from the transcription start site in the V region and therefore, leads to the selection of B cells with higher affinity for an antigen although the specificity will not change (Bransteitter *et al.*, 2006; Notarangelo *et al.*, 2006).

Cell to cell contact, such as the interaction between B and T lymphocytes, together with different cytokines plays a crucial role in the maturation of antibody responses

(Notarangelo *et al.*, 2006). Following antigenic stimulation, TD antigen is presented to T cells via class II MHC which leads to MHC-TCR interactions and the expression of CD40 ligand (CD40L or CD154) on T cells. CD40L is important in the induction of the CSR and is a member of the TNF superfamily (Sitati *et al.*, 2007). CD40L- CD40 interactions result in cross-linking of CD40 on the surface of B cells leading to B cell proliferation, generation of plasma cells, formation of germinal centres, and finally triggers CSR and SHM (Figure 4.2). T cells secrete cytokines such as IL-2, IL-4 and IL-10 which also promote B cell activation (Quezada *et al.*, 2004). Several signalling pathways are triggered by CD40L-CD40 interactions resulting in the activation of several genes responsible for B cell proliferation and antibody production (Thusberg & Vihinen, 2007). One of these genes is activation-induced cytidine deaminase (AID) which deaminates cytidine in DNA and has a crucial role in CSR and SHM (Imai *et al.*, 2003). Nuclear AID deaminates the V(D)J region and converts cytosine to uracil (Ta *et al.*, 2003). Uracil is then removed by uracil DNA glycosylase (UNG) in order to protect DNA from mutational damage which leads to an abasic site. As a result, this abasic site can be attacked by endonucleases generating DNA breaks which are the key for CSR (Imai *et al.*, 2003; Begum *et al.*, 2004). These signalling cascades also stimulate T cells to produce cytokines that determine the antibody class in B cells (Finkelman *et al.*, 1990).

Following ligation CD40 forms clusters at the B cell membrane and signalling pathways start with the interaction of the cytoplasmic domain with TNF receptor-associated factors (TRAFs) 2, 3 and 6 (Harigai *et al.*, 2004). This clustering and TRAFs initiate the signalling cascade involving nuclear factor (NF)- $\kappa$ B-inducing kinases, and protein serine/threonine kinases that activate transcription factors which regulate gene expression (Thusberg & Vihinen, 2007). In addition, TRAF-independent pathways may also be triggered following CD40L-CD40 interaction. For instance, Janus-associated kinases (JAKs) and signal

transducers and activators of transcriptions (STATs) might also be involved which are important in CD40-mediated functions (Hanissian & Geha, 1997). Isotype switching is also regulated by cytokines such as IL-4, IL-10, IL-13, IFN- $\gamma$  and TGF- $\beta$  (Tangye *et al.*, 2002). IFN- $\gamma$  has stronger effects on this process than IL-4 and both IFN- $\gamma$  and IL-4 are dominant over TGF- $\beta$  (Deenick, Hasbold & Hodgkin, 2005). In the presence of IL-4 and IL-13, CD40-stimulated B cells undergo isotype switching to IgG4 and IgE (Gascan *et al.*, 1991) or to IgG1 and IgG3 in the presence of IL-10 (Malisan *et al.*, 1996). Furthermore, IL-10 and TGF- $\beta$  are necessary for switching to IgA1 and IgA2 (Defrance *et al.*, 1992).



**Figure 4.2:** Schematic figure of CD40L-CD40 interaction. Activated T cells in germinal centre of lymph nodes interact with B cells and therefore, secrete interleukins (IL) which interact with IL receptors on B cells. As a result of CD40L-CD40 interaction, NFκB pathway is activated and as consequences AID and uracil N-glycosylase (UNG) are expressed which are crucial in CSR and SHM and therefore in isotype switching of antibody (Myers & Swanson, 2002).

#### **4.1.2: Complement Activation:**

An important component of both the innate and adaptive immune response is the complement system which is involved in both activation and regulation of the immune system (Nonaka & Miyazawa, 2002). The major functions of complement system are the recognition and elimination of pathogens through opsonisation and stimulation of phagocytosis to the immunoregulatory roles such as enhancing humoral immunity and modifying T cell immunity (Thurman & Holers, 2006).

The complement system contains approximately 35 heat-labile proteins that, once triggered, activate each other in a strict order (Nonaka & Miyazawa, 2002; Mueller-Ortiz, Drouin & Wetsel, 2004). It consist of three major pathways: the classical pathway, activated by antibodies; the lectin pathway, initiated by engagement of mannan-binding lectin; and the alternative pathway, triggered directly by surface molecules on a pathogen surface (Mueller-Ortiz, Drouin & Wetsel, 2004; Thurman & Holers, 2006).

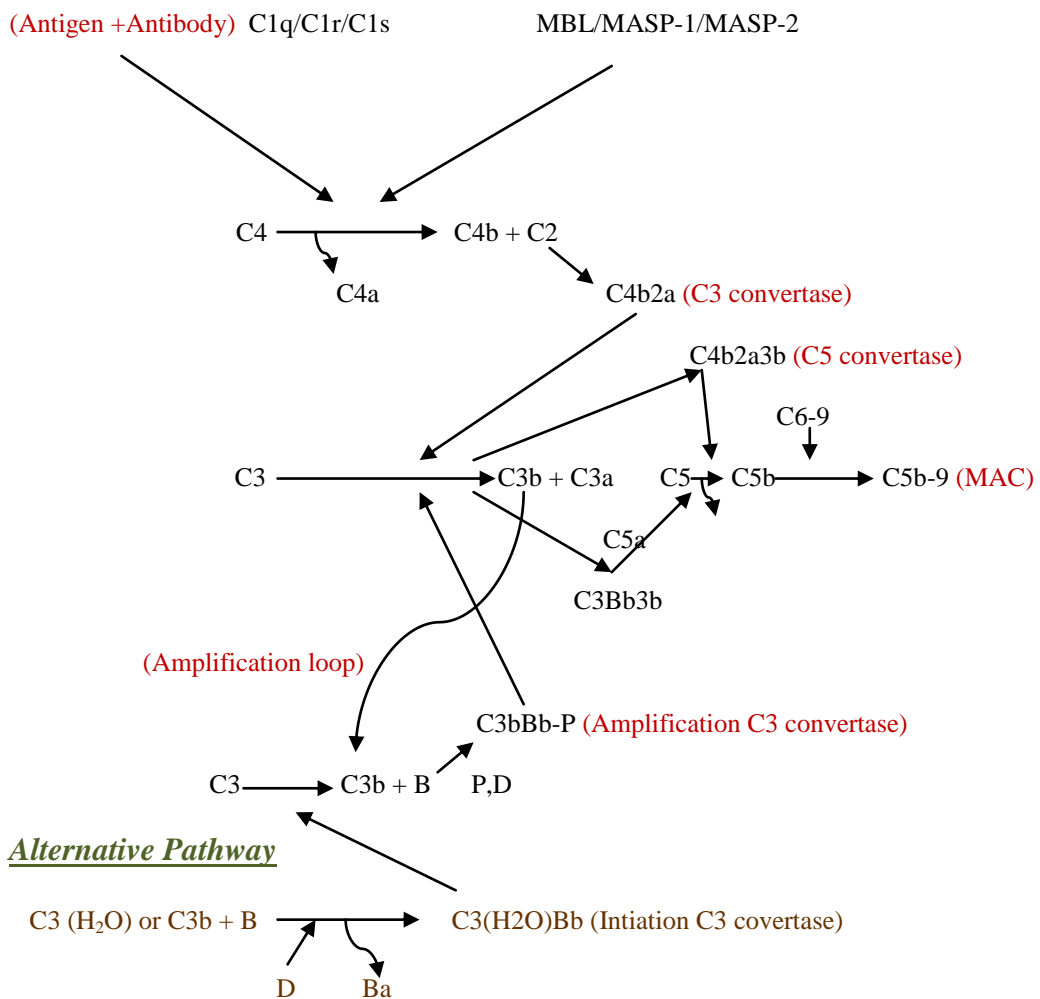
Some of the complement components are serine protease in nature which are crucial for the proteolytic activation of the whole system, among them C1r and C1s from the classical pathway and mannan-binding-protein-associated serine proteases (MASPs) from the lectin pathway (Cunningham & Quigg, 2005). They all share a common lytic pathway resulting in the formation of the membrane attack complex (MAC). At least 12 proteins are known to regulate and limit the complement activity and prevent sudden spontaneous complement activation which may lead to anaphylaxis (Cunningham & Quigg, 2005). Complement activation is also important for processing and limiting the size of circulating immune complexes (Alexander *et al.*, 2001) and therefore, transferring the immune complexes to Fc $\gamma$ R-bearing cells of the mononuclear phagocyte system.

#### **4.1.2.1: The Classical Pathway:**

The classical pathway is triggered by the binding of complement component C1q to complexes of IgG/antigen or IgM/antigen (Cunningham & Quigg, 2005). C1 contains 6 molecules of C1q, 2 molecules of C1r and 2 molecules of C1s. A binding site for C1q is present on the constant region of the antibodies. Activated C1 activates C4 and then C2, which form the C3 convertase (C4b2a) (Cunningham & Quigg, 2005). This catalyzes C3 to the C3a (anaphylatoxin) and C3b which binds to the growing immune complex. Following the formation of the C5 convertase (C4b2a3b), circulating C5 is cleaved into C5a (anaphylatoxin) and C5b. Similar to C3b, C5b joins to the immune complex, where it activates C6, C7, C8, and C9 and forms the C5b-9 complex (Figure 4.3). The hydrophobic domains of C7, C8, and C9 can insert into the lipid cell membranes of bacteria, erythrocytes, and nucleated cells and may lead to the changes in lipid layers of plasma membrane allowing the exchange of ions and water which results in the cell lysis. They may also activate a range of signaling pathways and induce the active process of endocytosis (Cunningham & Quigg, 2005; Wasowska *et al.*, 2007).

### Classical Pathway

### Lectin Pathway



**Figure 4.3:** Adopted figure of complement activation pathways. The classical, lectin, or alternative pathways can activate complement system. The proinflammatory mediators such as C3a and C5a, C3b can interact with complement receptors and the C5b-9 membrane attack complex (MAC). B, Factor B; D, Factor D; P, Properdin; MBL, mannose-binding lectin; MASP-1, MBL-associated serine protease-1; MASP-2, MBL-associated serine protease-2 (Cunningham & Quigg, 2005).

#### 4.1.2.2: The Alternative Pathway:

The complement system can also be activated spontaneously through the alternative pathway. Three unique components of the alternative pathway are factor B, factor D, and properdin which have crucial roles in this process (Thurman & Holers, 2006).

The alternative pathway is activated through a process referred as tickover of C3 (Thurman & Holers, 2006). As a result of the spontaneous hydrolysis of C3 in plasma, C3(H<sub>2</sub>O) is generated (Figure 4.3). C3(H<sub>2</sub>O) associates with Factor B resulting in the generation of C3(H<sub>2</sub>O)Bb. Factor B can be cleaved by an active serum protease, factor D, which leads to the generation of Ba and Bb. The Bb fragment remains with the complex and can cleave additional C3 molecules and therefore generate more C3Bb which is a C3 convertase. The serum protein properdin (P) stabilizes protein-protein interactions during the process. The “amplification loop” of the alternative pathway can also be initiated when C3b, generated by classical or the lectin pathway, binds factor B which leads to the process as described above. The C3 and C5 convertases of the alternative pathway (C3bBb and C3bBb3b) also trigger and generate C3a, C3b, C5a, and C5b-9 (Cunningham & Quigg, 2005; Thurman & Holers, 2006). Since the alternative pathway is able to be activated spontaneously, it requires continuous active control by cell-bound complement regulatory proteins (CRP) (Rose *et al.*, 2008). CRP may influence the complement activation cascades resulting in the prevention of C1 activation by C1 inhibitor and blocking formation of C5b-9 by CD59.

There is a further group of proteins which are contained within the regulators of complement activation gene family and these include the C4-binding protein, factor H, decay accelerating factor (DAF; CD55), membrane co-factor protein (CD46), and complement receptor 1 (CR1; CD35) (Cunningham & Quigg, 2005). In humans DAF and CR1 lower C3 and C5 activation and CD59 restricts C5b-9 formation.



#### **4.1.2.3: The Lectin Pathway:**

The lectin pathway plays a crucial role in innate immunity particularly between the first 6 and 18 months of age when the adaptive immune system is still immature (Turner, 2003). This pathway is an antibody-independent pathway triggered by the binding of mannose-binding lectin (MBL) ficolins to a range of sugars including N-acetyl-D-glucosamine, mannose, N-acetyl-mannosamine, fucose and glucose found on the surface of microorganisms (Krarup *et al.*, 2007; Takahashi *et al.*, 2007). MBL is structurally similar to C1q, and activates the complement system through MBL-associated serine protease (MASP) which is similar to C1r and C1s of the classical pathway (Nonaka & Miyazawa, 2002). The binding of ficolin and MASPs molecules to pathogen surface leads to conformational changes in these complexes resulting in autoactivation of the MASPs which initiates antibacterial activity by the terminal, lytic complement components.

Three types of MASPs, referred to as MASP-1, MASP-2 and MASP-3 have been identified. MASP-2 can autoactivate and cleaves C4 and C2 to make a C3 convertase, C4b2a. MASP-1 has an ability to cleave C3 directly (Takahashi *et al.*, 2007).

#### **4.1.2.4: IgG subclasses and the Complement System:**

Although the C domains of IgG subclasses share almost identical amino acid sequences, their ability to activate complement are different (Tan *et al.*, 1990; Sitaru, Mihai & Zillikens, 2007). Various studies have shown that IgG3 is the most efficient at activating complement followed by IgG1, with IgG2 being a weak activator, whilst IgG4 seems to be unable to activate complement (Doekes, van Es & Daha, 1984; Garred, Michaelsen & Aase, 1989; Saeland *et al.*, 2003; Sitaru, Mihai & Zillikens, 2007). Some of the other

factors which may affect complement activation are the segmental flexibility of the hinge region (Redpath *et al.*, 1998; Hezareh *et al.*, 2001), antigen specificity and density (Da Silveira *et al.*, 2002) and high antibody concentration (Bard *et al.*, 2003).

It has been suggested that the hinge region determines the difference between IgG isotypes partly since the hinge regions have diverse sequences (Tan *et al.*, 1990) and the IgG3 hinge has the highest flexibility (Redpath *et al.*, 1998). In addition the density and distribution of antigen epitope influences C1 activation by creating a suitable angle of the IgG Fab arms (Lucisano Valim & Lachmann, 1991; Da Silveira *et al.*, 2002). Complement activation through the classical pathway is also triggered more efficiently at high antibody concentration which is markedly influenced by antibody affinity (Bard *et al.*, 2003).

IgG1 activates C4 and C3 better than IgG3 and IgG2 at high antigen concentrations whilst at low epitope density IgG3 is the best inducer of complement activation followed by IgG1 and IgG2. On the other hand, at high epitope density and complement concentration IgG2 is able to trigger the alternative pathway efficiently in contrast to IgG1 and IgG3 (Garred, Michaelsen & Aase, 1989; Lucisano Valim & Lachmann, 1991; Sitaru, Mihai & Zillikens, 2007).

#### **4.1.3: Hypersensitivity:**

The term of hypersensitivity is used to describe exaggerated or inappropriate immune responses to an allergen or antigen that result in tissue damage.

Hypersensitivity reactions have been classified to 5 types as follows:

- Type I or immediate hypersensitivity which develops when antigen-antibody complexes, in this case IgE, cause the degranulation of mast cells with the release of histamine and other mediators (Marshall & Bienenstock, 1994; Wigginton *et al.*, 2008). Anaphylaxis and hay fever are the examples of this type of hypersensitivity reaction.
- Type II or antibody-mediated hypersensitivity involves IgG or IgM bound to the antigens with subsequent complement fixation (Jewett *et al.*, 1997; Niwa *et al.*, 2005). Examples of this mechanism are Haemolytic Anaemia, Good Pasture's Syndrome and Rheumatic Fever.
- Type III or immune complex-mediated hypersensitivity (described in section 4.1.3.1)
- Type IV or delayed-type hypersensitivity (described in section 4.1.3.2)
- Type V or stimulatory hypersensitivity in which antibody (IgG) binds to the cell surface receptors which mimic the effects of the ligand and have a stimulatory effect on its target. This type is seen in Myasthenia gravis, Graves disease and Thyrotoxicosis due to TSH autoantibodies (Rajan, 2003).

HP is considered to be a mixture of type III and Type IV responses (Yi, 2002) which I will discuss further.

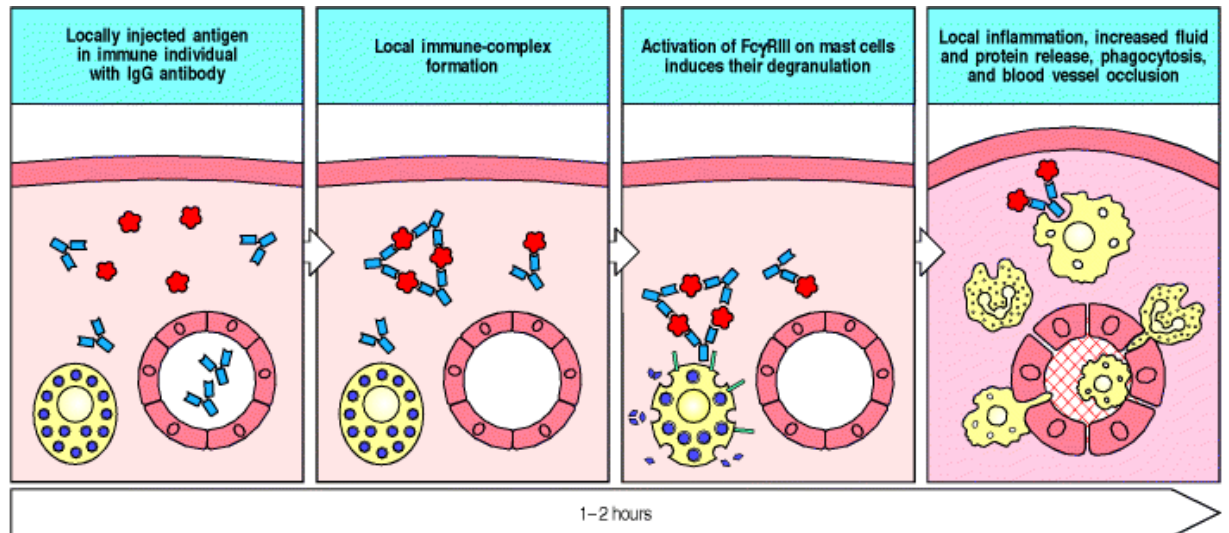
#### **4.1.3.1: Type III Hypersensitivity Reaction:**

Type III hypersensitivity reactions can be triggered by exposure to soluble antigens and clinical presentation may happen after 3-10 hours. The pathology starts by the deposition of IC at the basement membrane of blood vessels, kidneys or in the lungs in the case of HP. IC then can bind to FC receptors on leukocytes, leading to tissue injury. The pathogenic potential of IC is determined by their size and concentration, affinity, and isotype of the antibody in the IC. Clinically type III hypersensitivity reaction has 2 types of presentation including localized and generalized types (Riedl & Casillas, 2003; Ghaffar, 2004; Ellsworth *et al.*, 2008).

The arthus reaction is an example of a localised reaction. Following injection an antigen into the skin of a sensitized individual, circulating IgG antibody forms IC locally which can bind to FC $\gamma$  RIII (CD16) on mast cells and other leukocytes. This initiates degranulation of mast cells and a local inflammatory response with increased vascular permeability which leads to the retention of fluid and influx of leukocytes to the site (Figure 4.4.a) (Razin, Pecht & Rivera, 1995). These IC can also activate complement through the classical pathway. C3a and C5a not only cause localized mast cell degranulation and increased vascular permeability but also can bind to C5a receptors on leukocytes.

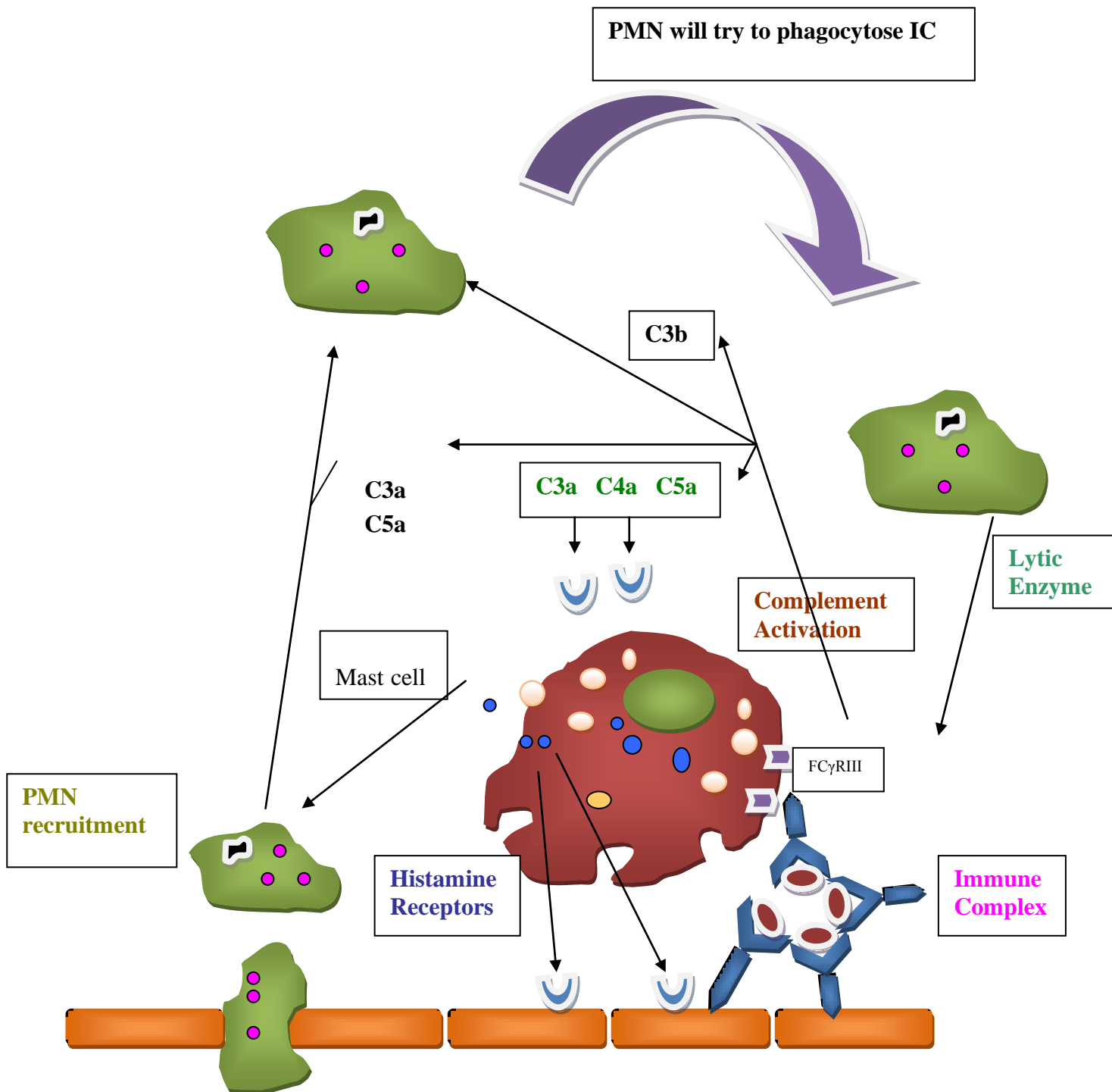
On the other hand, the systemic type of reaction can be described in serum sickness as a result of the injection of large quantities of foreign soluble antigens and deposition of IC throughout the body. The clinical features are chills, fever, rash, arthritis, and glomerulonephritis. The ligation of IC with FC $\gamma$  RIII triggers mast cells degranulation (Figure 4.4.b) (Ellsworth *et al.*, 2008). These IC also activate complement and bind to leukocytes bearing Fc and complement receptors which can cause widespread tissue injury.

Anti-lymphocyte globulin, which is an immunosuppressive agent used in transplant recipients, can cause serum sickness (Cuzic *et al.*, 2001).



**Figure 4.4.a:** The local type III hypersensitivity reaction (Arthus Reaction).

Following injection of the antigen into the skin of sensitized individual and formation of immune complexes, they bind to  $\text{Fc}\gamma\text{RIII}$  on mast cells and other leukocytes which initiates mast cell degranulation and increased vascular permeability. This leads to the retention of fluid and influx of leukocytes to the site (Janeway, 2008).



**Figure 4.4.b:** Development of systemic type III hypersensitivity reaction. Immune complexes initiate the complement cascade. The complement intermediates stimulate (i) mast cell degranulation, (ii) neutrophil recruitment, and (iii) releasing lytic enzyme from neutrophil in order to help in phagocytosis of C3b-coated immune complexes.

#### **4.1.3.2: Type IV Hypersensitivity Reaction:**

Type IV hypersensitivity reactions are mediated by antigen-specific effector T cells and are an important mechanism in host defence against intracellular bacteria and parasites. It has 2 phases including the sensitisation phase and effector phase.

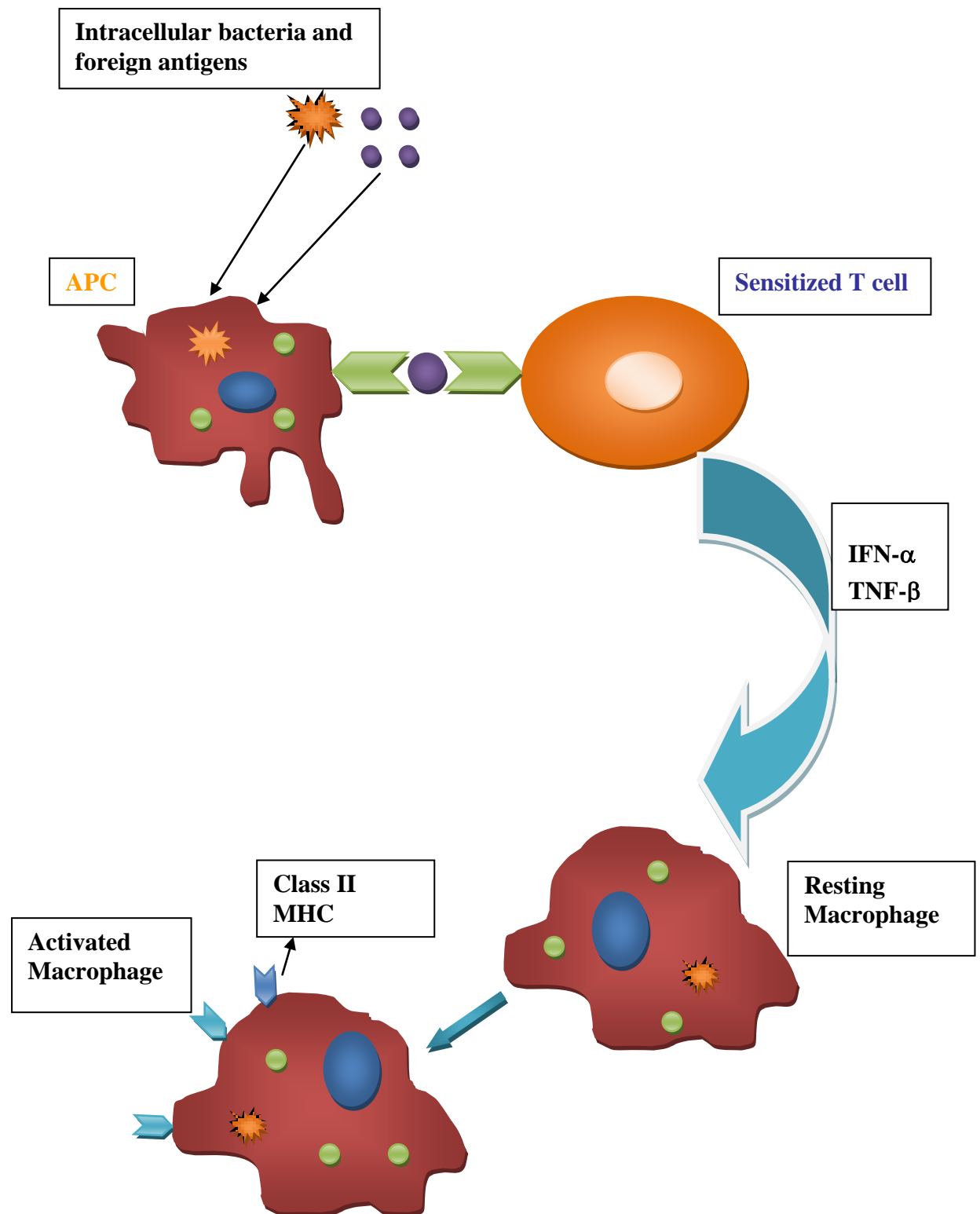
As seen in cutaneous hypersensitivity, the sensitizing chemicals react with self proteins and create protein-hapten complexes which bind to MHC class II molecules which are recognised as foreign antigens by T cells. These antigens are processed by cutaneous Langerhans' cells during the sensitization phase and migrate to regional lymph nodes to activate T cells and generate memory T cells. The activated T cells are mostly CD4<sup>+</sup> and Th1 cells but CD8<sup>+</sup> T cells may also be activated (Czarnobilska, Obtulowicz & Wsolek, 2007).

Following further exposure, the antigen is presented to memory T cells in the dermis which triggers the production of T cell cytokines such as IFN- $\gamma$  and IL-17. This stimulates the keratinocytes of the epidermis and they release cytokines such as IL-1, IL-6, TNF- $\alpha$  and GM-CSF (Squier & Cohen, 1994; Kinet, 1999). These cytokines activate resting macrophages and increase their phagocytic potential. These activated macrophages also express higher level of class II MHC molecules, and therefore have more effective function as APCs (Figure 4.5.a). In the case of EAA, the release of the lytic enzymes from activated macrophages can initiate non-specific tissue damage and create granulomatous lesions (Yi, 2002).

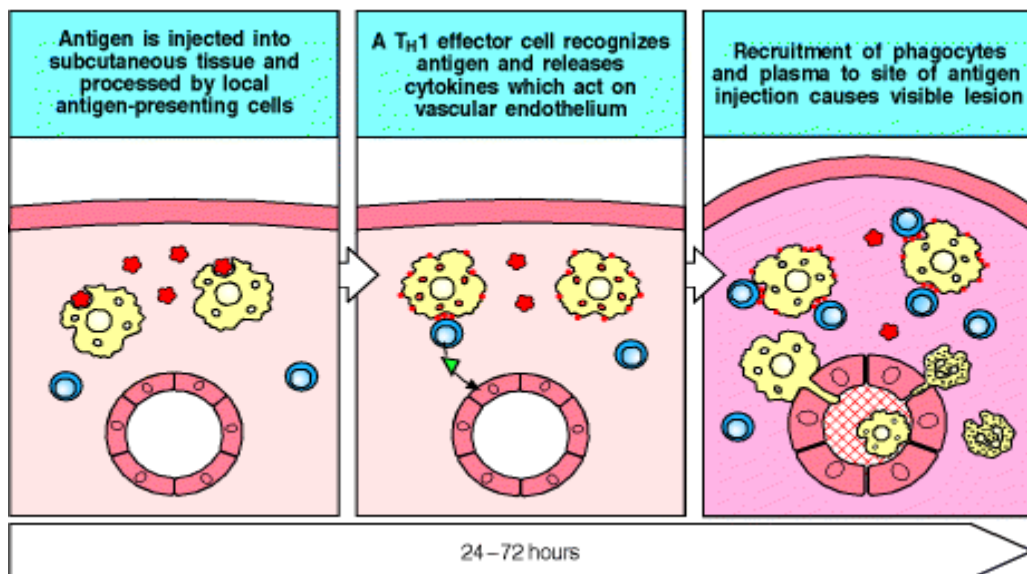
Another example of type IV hypersensitivity is seen after immunization with BCG when a local T cell-mediated inflammatory reaction begins over 24–72 hours (Figure 4.5.b) (Umemura *et al.*, 2007). Following injection complexes of peptide/MHC class II molecules on APCs are recognised by Th1 cells leading to release of inflammatory cytokines such as



IFN- $\gamma$  and TNF- $\beta$ . The cytokines can increase local vascular permeability which causes a visible swelling at this site. This process takes 24-48 hours to develop.



**Figure 4.5.a:** Type IV hypersensitivity reaction. During the first 1-2 weeks following primary contact between antigen (ie: intracellular bacteria, chemicals) and antigen presenting cells (APCs, ie: macrophage), T helper cells proliferate. After secondary contact with antigen, T cells secrete a variety of cytokines which lead to activation of macrophages. These cells express higher level of class II MHC molecules and increased level of phagocytosis.



**Figure 4.5.b:** The local presentation of type IV hypersensitivity. Following subcutaneous injection of the antigens, APCs pick up the antigens and present them via class II MHC to Th1 cells. This stimulates Th1 cells to release cytokines which cause an increase in vascular permeability and therefore swelling at the site of injection (Janeway, 2008).

#### **4.1.4: Evidence of Type III and IV Hypersensitivity reactions in PFL:**

As previously described (sections 4.1.3.1, 4.1.3.2), the acute form of disease usually occurs 4-6 hours after exposure to the relevant antigen in susceptible individuals (Kaltreider, 1993; Bourke *et al.*, 2001). Basically alveolar macrophages engulf foreign antigens, transport them to the cell surface and present the complex of MHC/antigen to Th cells (Kaneko *et al.*, 2000; Desombere *et al.*, 2005). This leads Th cells to become activated and interact with B cells. As a consequence, B cells start differentiating into plasma cells which secrete antibodies (Jenkins *et al.*, 2001). The reaction between antigen and antibody produces IC, which may become deposited in the basement membrane of the lungs and these will then activate the complement cascade by the classical pathway (Cunningham & Quigg, 2005; Wasowska *et al.*, 2007). Neutrophils attracted to the site of inflammation try to phagocytose the IC and also release lytic enzymes causing localized tissue damage and neutrophilic alveolitis (Razin, Pecht & Rivera, 1995).

These findings support the initial report of HP being an immune complex mediated (Hypersensitivity reaction type III) disease but subsequently studies have shown that T cells and inflammatory cytokines may have even greater roles to play in the pathogenesis of disease than immune complexes (Curtis *et al.*, 1991; Ohkawa *et al.*, 2001; Curtis *et al.*, 2002). This is supported by adoptive transfer of experimental HP by intravenous injection of a Th1 cell line in animal models (Schuyler *et al.*, 1997).

During the chronic phase of HP, activated CD4<sup>+</sup> T cells can be divided into either Th1 or Th2 cells based on their pattern of cytokine secretion (Irifune *et al.*, 2003; Barrera *et al.*, 2008). Th1 cells secrete IL-2, IFN- $\gamma$  and TNF- $\beta$  which help in activating resting macrophages and their phagocytic activities which are responsible for cell mediated immunity reactions. Lytic enzymes from activated macrophages can cause a non-specific tissue damage and the formation of granulomatous lesions (Kinet, 1999). IL6 and TNF- $\alpha$

also are higher in the chronic stage of disease as compared to the acute stage and this emphasizes the importance of these cytokines in the development of pulmonary fibrosis (Ohtani *et al.*, 1999).

#### **4.1.5: Pigeon Intestinal Mucin as an Antigen in PFL:**

PIM is a complex high molecular weight glycoprotein with a high carbohydrate composition of 70-80% (Hounsell, Davies & Renouf, 1996; Baldwin *et al.*, 1999; Thornton & Sheehan, 2004).

PIM is produced by goblet cells in the intestine of pigeon and has been shown to be highly antigenic in humans (Todd, Coan & Allen, 1991). It has been detected in pigeon droppings and pigeon bloom collected from pigeon lofts (Fredricks, 1978; Todd, Coan & Allen, 1991; Baldwin *et al.*, 2000b) and thus pigeon fanciers have been shown to be exposed to high levels of the antigen. The carbohydrates in pigeon intestinal mucin may be O or N-linked with the majority being O-linked. Based on the resistance to proteolytic digestion, there are 2 regions on the mucin protein core: the highly glycosylated (bottle brush) region which is shown in figure 4.6.a and the regions susceptible to proteolysis where glycosylation is little or absent (Baldwin *et al.*, 1999). Following proteolysis, the heavily glycosylated and antigenic mucin fragments are produced which are resistant to further breakdown, thus they may persist in the pigeon loft for long periods of time. These highly glycosylated regions are rich in serine, threonine and proline which are the sites for sugar attachment. The glycosylation of PIM was investigated by enzyme-linked lectin assay (ELLA). High levels of  $\alpha$ -2, 3 N-acetyl neuraminic acid ( $\alpha$ -2, 3 NeuNAc), N-acetyl galactosamine (GalNAc) and N-acetyl glucosamine (GlcNAc) and low levels of fucose (Fuc) and galactose (Gal) were detected. Very high levels of  $\alpha$ -2, 3 NeuNAc in PIM

[illegible]

GalNAc(β1-3)GalcpNAc(β1-4)Gal(β1-3)Gal(α/β)  
 ↑  
 3  
 Fucα1

114

suggest that a large proportion of the oligosaccharide chains are sialylated (Baldwin *et al.*, 1999; Thornton & Sheehan, 2004). The terminal structure of these side chains are shown in figure 4.6.b (Baldwin *et al.*, 2005).

The antibody positive fanciers have high titres of anti-mucin IgG (Todd, Coan & Allen, 1991; Baldwin *et al.*, 1998a; Baldwin *et al.*, 1998c). Although IgG titres are similar in symptomatic and asymptomatic fanciers, a significant difference in the IgG subclass against PIM has been identified. All 4 subclasses of IgG antibody against PIM are present in the pigeon fanciers' serum, however, it has been demonstrated that anti-PIM IgG1 is significantly higher in symptomatic individuals and this correlates with the development of disease (Baldwin *et al.*, 2000b). It has been suggested that the immune complexes containing IgG1 are able to bind to a range of FC $\gamma$  receptors more effectively than the immune complexes containing other IgG antibodies and thus they may have more significant pathological effects (Burton, Gregory & Jefferis, 1986; Todd, Coan & Allen, 1993; Baldwin *et al.*, 1998c). Furthermore, IgG2 levels are high in both symptomatic and asymptomatic fanciers whilst IgG3 and IgG4 are much lower in both groups (Baldwin *et al.*, 1998a; Baldwin *et al.*, 1998c; Baldwin *et al.*, 2000a).

High levels of anti- proteolytically digested mucin- IgG1 and IgG2 have been detected, suggesting that these antibodies are carbohydrate-specific. On the other hand, IgG3 reactivity was significantly reduced in proteolytically digested mucin, suggesting that IgG3 reacts against the protein portion of the molecule (Baldwin *et al.*, 1999).

Thus, PIM may stimulate both T-dependent (TD) and T-independent (TI) antibody responses due to the highly glycosylated nature of this molecule. TI responses are found against carbohydrate (CHO) antigens whilst TD responses are typically found against protein antigens (Obukhanych & Nussenzweig, 2006). Interestingly, in this case IgG1 is generated against the CHO region of the PIM, which is an unusual observation as generally

IgG1 is considered to be a TD antibody and is almost exclusively active against protein antigens whilst IgG2 is a TI antibody and is produced against carbohydrate antigens (Hammarstrom & Smith, 1986).

#### **4.1.6: Antibody Affinity:**

The strength of binding between a monovalent antibody molecule and an antigenic determinant is called the affinity (Steward, 1979; Rosleos *et al.*, 2007). It is under polygenic control and is important in providing an effective and protective immunity. The fit of the antigen into the binding groove of a Fab, the size of contact area, hypervariable loop conformation changes (Rini, Schulze-Gahmen & Wilson, 1992), rotation of  $V_H$ - $V_L$  interface (Stanfield *et al.*, 1993) and the distribution of hydrophobic groups influence the antibody affinity. The reduced flexibility of the antibody binding pocket has also been reported to be important in the generation of high affinity antibody (lock-and-key model) (Thorpe & Brooks, 2007).

On the other hand, functional affinity or avidity is defined as the multivalent binding of an antibody to a complex antigen and shows the overall binding strength in an immune complex. Increasing antibody affinity over the lifetime of an immune response is referred to as affinity maturation and depends on SHM in the variable region of the antibody molecule mainly in the CDR which increases the surface complementarity (Furukawa *et al.*, 2001; Acierno *et al.*, 2007). It is triggered when an antigen enters the body and is presented to T cells via class II MHC which leads to MHC-TCR interaction and expression of CD40L on T cells. CD40 cross-linking on the surface of B cells leads to B cell proliferation, generation of plasma cells and also triggers CSR and SHM which previously



discussed in section 4.1.1.3 (Imai *et al.*, 2003; Zarrin *et al.*, 2005; Notarangelo *et al.*, 2006). Since low affinity antibody may not be able to eliminate the antigen effectively, the formation of immune complexes in an antigen excess situation may cause immune complex disease (Devey, Beckman & Kemeny, 1993). On the other hand, it has been suggested that infection with high antigen load even in the presence of high level of high affinity antibodies may result in the deposition of immune complexes (Steward, 1979).

The structural difference in the constant region of the antibodies may also determine the affinity of the antibody (McCloskey *et al.*, 1996). Carbohydrate groups on antibodies also have important roles in solubility, stability, immune complex clearance, antibody dependent cellular cytotoxicity and macrophages recognition through FC Receptor. For instance, the absence of glycosylation, mutation in the COOH-terminal of HC region and fucose depletion can influence the antibody affinity (Bazin *et al.*, 1992; Okazaki *et al.*, 2004).

#### **4.2: Aims:**

The aim of this section was to study antibody responses in symptomatic and asymptomatic pigeon fanciers and to identify the functional affinity of specific anti-pigeon antigen IgG isotypes.

#### **4.3: Results:**

##### **4.3.1: Human Subjects:**

Human subjects were classified into one of the following 5 groups based on clinical findings and IgG titres of equal or greater than 10000 as measured by ELISA. It has been shown that an ELISA titre of 10000 or more correlated with the detection of precipitating antibodies as measured by CIE (Baldwin personal communication). 4 of these groups were pigeon fanciers and one group was control negative individuals:

- Group A: Symptomatic with ELISA titres  $\geq 10000$  (n: 25)
- Group B: Asymptomatic with ELISA titres  $\geq 10000$  (n: 25)
- Group C: Symptomatic with ELISA titres  $< 10000$  (n: 25)
- Group D: Asymptomatic with ELISA titres  $< 10000$  (n: 25)
- Group E: Control negative with no pigeon contact with ELISA titres  $< 1000$  (n: 10)

Table 4.3 shows the details of the background of individuals in group A-D.

	Group A	Group B	Group C	Group D	Group E
<b>Age (mean)</b>	50	50	46	40	34
<b>(range)</b>	(21-73)	(25-77)	(23-77)	(14-60)	(24-47)
<b>Sex (number)</b>					
• <b>Male</b>	23	25	25	25	8
• <b>Female</b>	2	0	0	0	2
<b>Years spent with pigeon (mean)</b>	29	33	24	20	0
<b>Number of pigeons (mean)</b>	83	87	101	74	0
<b>Hours of contact/wk (mean)</b>	25	29	23	29	0
<b>Smoking</b>					
• <b>Non/Ex-smoker</b>	22	24	13	7	8
• <b>current smoker</b>	3	1	12	18	2

**Table 4.3:** Details of the human subjects classified in group A-E.

There were no significant age differences between groups A and B, and group C and D. All fanciers included in this study were males except 2 females in group A. Group B had the longer history of contact with pigeons than group A, however, the difference was not significant. This difference was not also significant when group C was compared with group D.

When smoking habit of fanciers was compared between groups, there was no significant difference between group A and B, and group C and D.

#### **4.3.2: Preparation of Antigens:**

##### **4.3.2.1: Pigeon Intestinal Mucin (PIM):**

Soluble mucin was prepared as described in section 3.1. The results of the first and the second CsCl density gradient centrifugations are shown in figure 4.7.

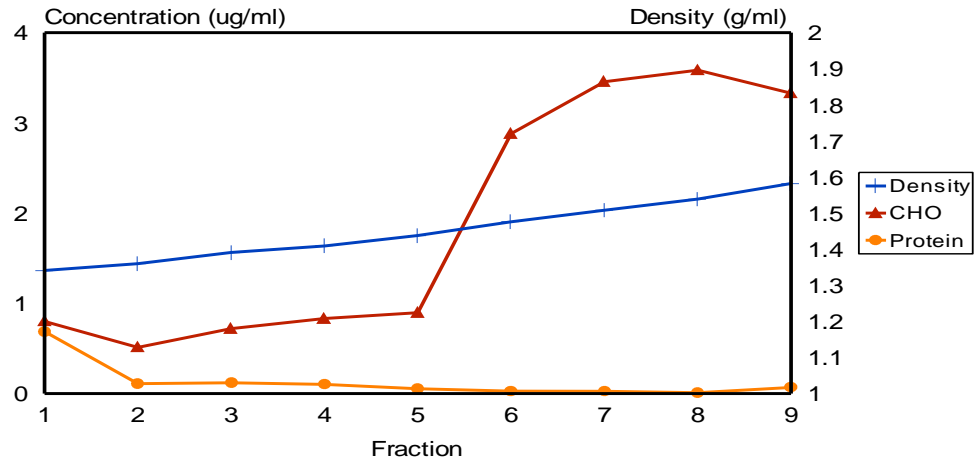
After the first CsCl density gradient centrifugation, high concentrations of protein were found in the earlier, low density fractions and low concentrations of protein were detected in the later fractions (4.7.A).

On the other hand, high concentrations of carbohydrate were found in the later, high density fractions, whilst low concentrations of carbohydrate were detected in the earlier fractions (4.7.A).

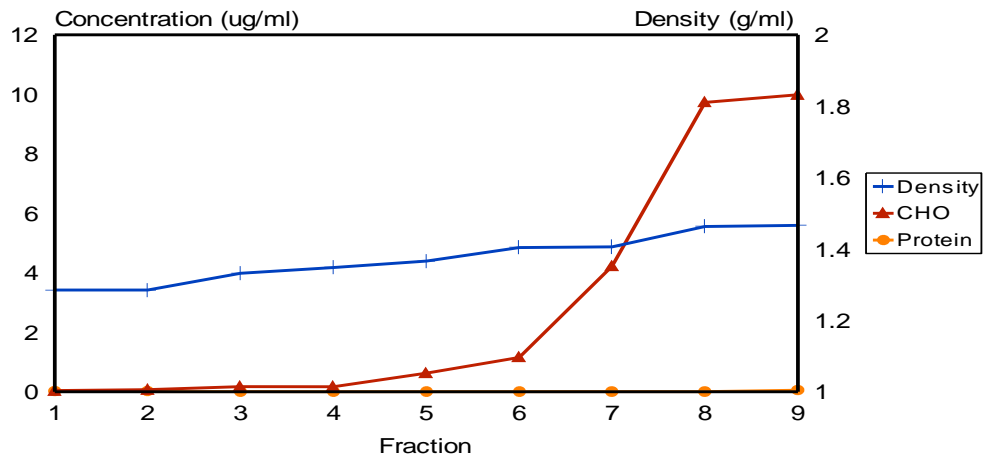
Fractions 6-9 (density: 1.476-1.582 g/ml) of the first CsCl density gradient centrifugation had the highest concentrations of carbohydrate and were pooled and underwent a second run of centrifugation. After the second CsCl centrifugation, the highest concentrations of carbohydrate were recovered from fractions 6-9 (density: 1.40-1.466 g/ml) and very little protein was present in any of the removal fractions (4.7.B).

Fractions 6-9 were pooled and designated as PIM, freeze dried and used for future studies.

A



B



- (A) Carbohydrate (CHO) and protein concentration ( $\mu\text{g/ml}$ ) and density (g/ml) after the first CsCl centrifugation. Fractions 6-9 (density: 1.476-1.582) had the highest concentrations of CHO.
- (B) CHO and protein concentrations ( $\mu\text{g/ml}$ ) and density (g/ml) after second CsCl centrifugation. Fractions 6-9 (density: 1.40-1.466) had the highest concentrations of CHO.

#### **4.3.2.2: Trypsinised Mucin:**

TM was prepared as described in section 3.2. The final mixture was aliquoted and stored at  $-20^{\circ}\text{C}$ .

#### **4.3.2.3: Fresh Pigeon Droppings (PDF) and Old Pigeon Droppings (PDO):**

Fresh (PDF) and old (PDO) pigeon droppings had previously been collected from a pigeon loft (supplied by Dr Christopher Baldwin). They were prepared as described in section 3.3. A proportion of freeze-dried extracts were reconstituted in deionised water to make PDF and PDO at 1 mg/ml concentration for further experiments.

#### **4.3.2.4: PIS:**

The supernatant of the pigeon intestinal scrapings was prepared as described in section 3.1 and used as one of the pigeon antigens called PIS. This antigen contains soluble mucus and is different from PIM with respect to the high concentration of protein in this sample.

#### **4.3.2.5: Pigeon serum:**

PS was commercially supplied by Sigma (Sigma, UK).

### **4.3.3: Measurement of Protein and Carbohydrate concentrations of Pigeon Antigens:**

The carbohydrate and protein concentrations of each pigeon antigen were measured by the PAS and Bio-Rad methods as described previously (sections 3.5 and 3.6).

The results are presented in table 4.4. PIS had the highest (4480  $\mu\text{g/ml}$ ) carbohydrate concentration whilst mucin and PDO had a much lower (280  $\mu\text{g/ml}$ ) carbohydrate concentration. On the other hand, mucin and TM had the lowest ( $<5$   $\mu\text{g/ml}$ ) protein concentration whilst PS contained the highest (640  $\mu\text{g/ml}$ ) concentration of protein.

Pigeon Antigens	Protein Concentration (µg/ml)	Carbohydrate Concentration (µg/ml)
<b>Mucin</b>	< 5	280
<b>TM</b>	< 5	400
<b>PDF</b>	240	1600
<b>PDO</b>	10	280
<b>PIS</b>	400	4480
<b>PS</b>	640	1600

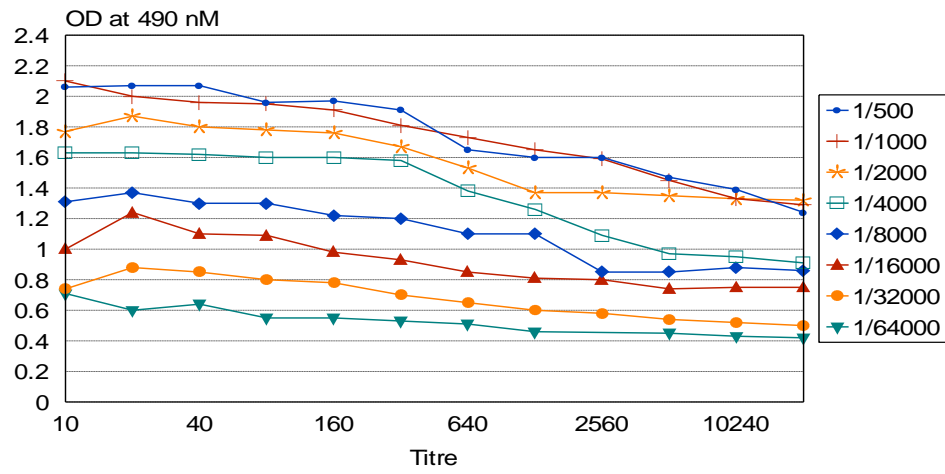
**Table 4.4:** The protein and carbohydrate concentrations of the pigeon antigens used in this study.

#### **4.3.4: Checker Board ELISA:**

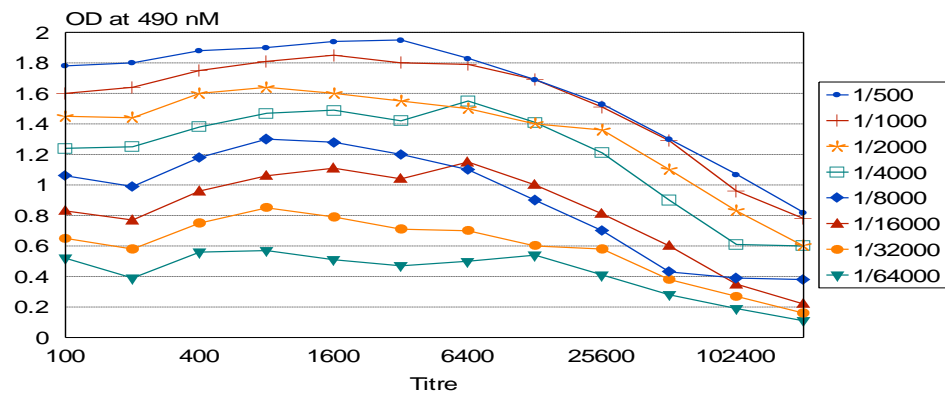
To establish the optimal concentration and dilution of each pigeon antigen a checkerboard ELISA was performed as described in section 3.8.

Examples of individual antigen checker board for PDO, PIS and PIM can be seen in figures 4.8- 4.10. Data for PDF, PS and TM are not shown. The optimal antigen concentration for coating the ELISA plates for each antigen is shown in table 4.5.

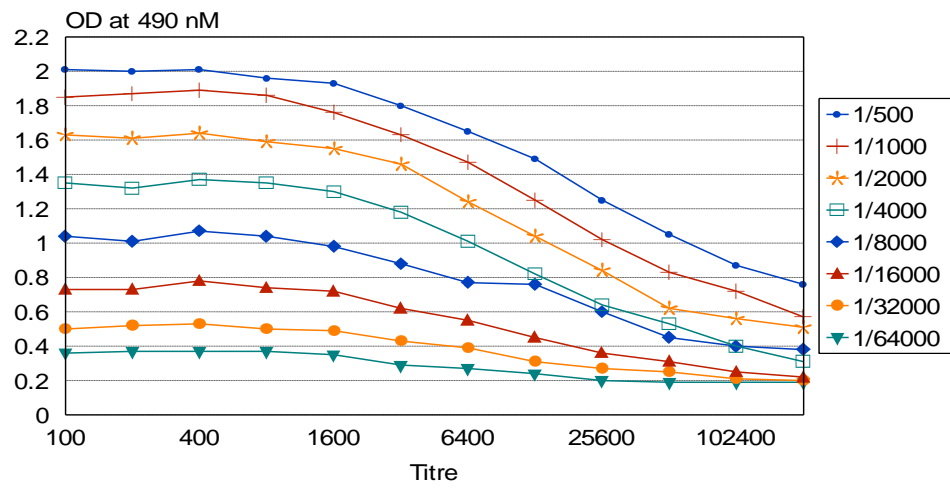




**Figure 4.8:** The checker board ELISA graph for PDO, the optimal dilution of PDO was 1/500.



**Figure 4.9:** The checker board ELISA graph for PIS, the optimal dilution of PIS was 1/7000.



**Figure 4.10:** The checker board ELISA graph for PIM, the optimal dilution of PIM was 1/2000.

<b>Pigeon Antigen</b>	<b>Optimal dilution for ELISA</b>	<b>[CHO] µg/ml at the optimal dilution</b>	<b>[protein] µg/ml at the optimal dilution</b>
<b>PIM</b>	1/2000	0.14	0.003
<b>TM</b>	1/500	0.8	0.01
<b>PS</b>	1/2000	0.8	0.32
<b>PDF</b>	1/1000	1.6	0.24
<b>PDO</b>	1/500	0.6	0.02
<b>PIS</b>	1/7000	0.64	0.06

**Table 4.5:** The optimal dilution and concentration of pigeon antigens as determined by checker board ELISA for coating ELISA plates.

The ELISA plates were coated with the pigeon antigens with carbohydrate concentrations of 0.14- 0.8 µg/ml. The diluted PIM used for coating the ELISA plates had the lowest carbohydrate concentration 0.14 µg/ml whilst the highest concentration belonged to PS and TM (0.8 µg/ml). The protein concentration of the coating antigens varied 0.003 µg/ml (PIM) to 0.24 µg/ml (PDF).

#### **4.3.5: IgG ELISA:**

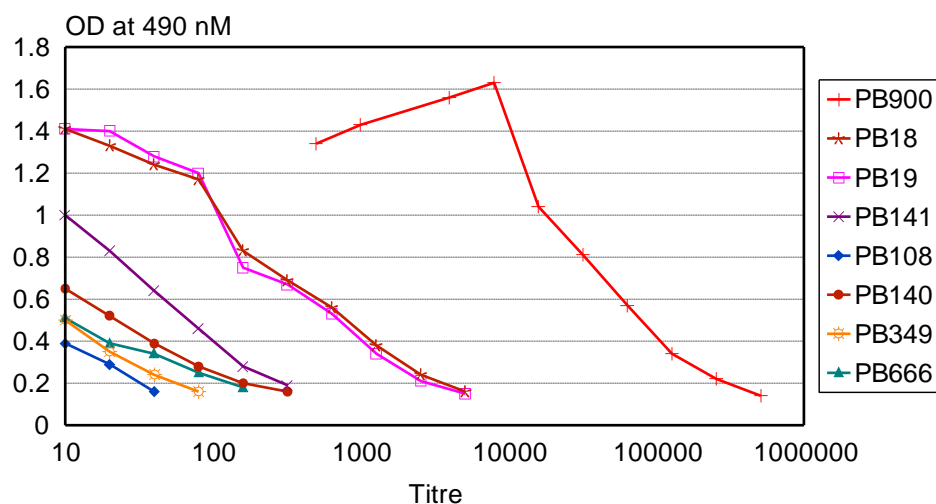
ELISA was used to determine the titre of IgG against the pigeon antigens including PIM, TM, PS, PDF, PDO and PIS as previously described in section 3.9.

Absorbance at 490 nM was plotted against the reciprocal of the serum dilution.

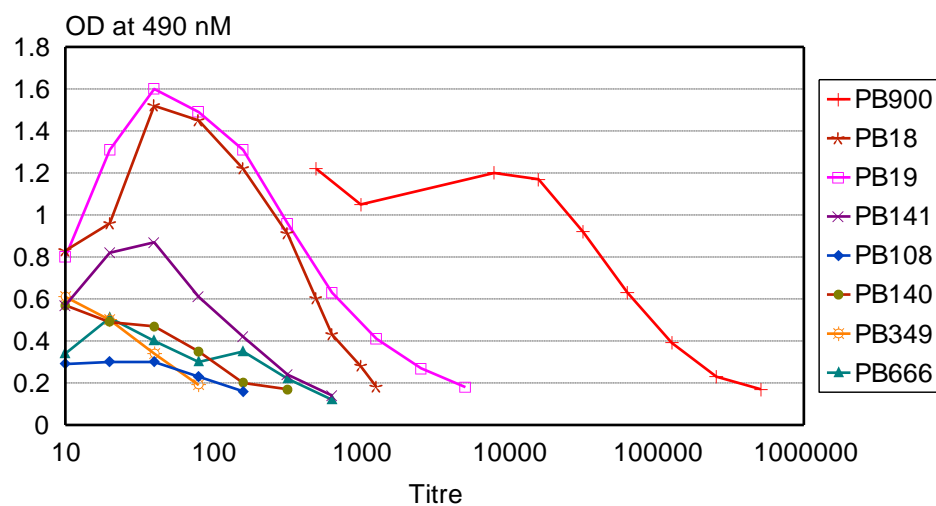
The reciprocal of the dilution of each serum sample that gave an optical density of 0.2 (approximately 3 times of the background) was considered to be the end point titre of the test serum. An example of an ELISA test plate for each antigen is shown in figures 4.11- 4.16.

Note that as the samples were tested in different ELISA plates on different days, the same positive control serum sample was always included on each plate. This enables the results from different plates on different days to be compared. The positive control sample was from an asymptomatic fancier with high specific antibody titres against pigeon antigens.

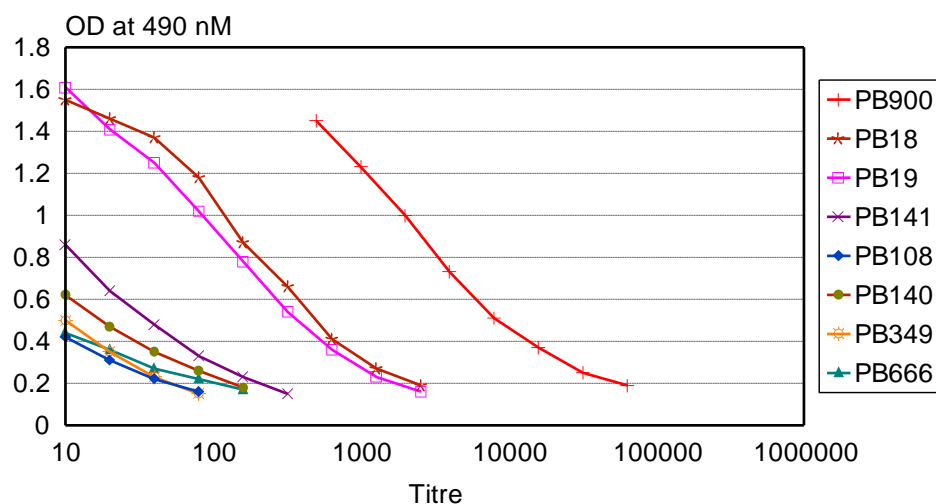
The median and the range of IgG titres for each clinical group are shown in Table 4.6 and figure 4.17 shows the level of IgG against all pigeon antigens in the 5 study groups.



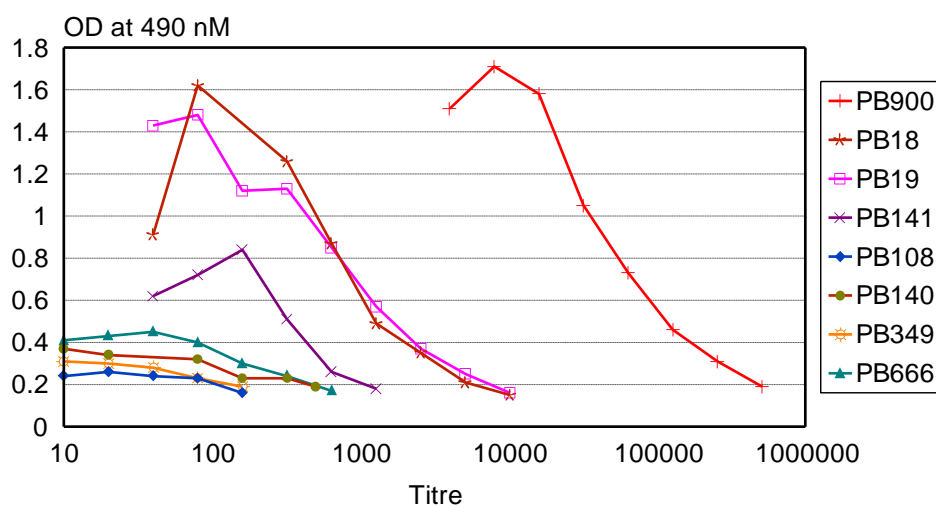
**Figure 4.11:** Anti-mucin IgG graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



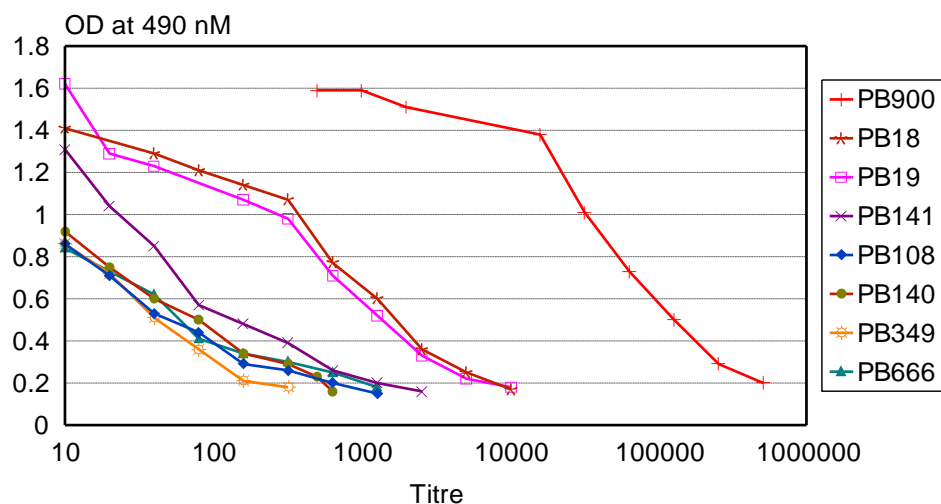
**Figure 4.12:** Anti-TM IgG graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



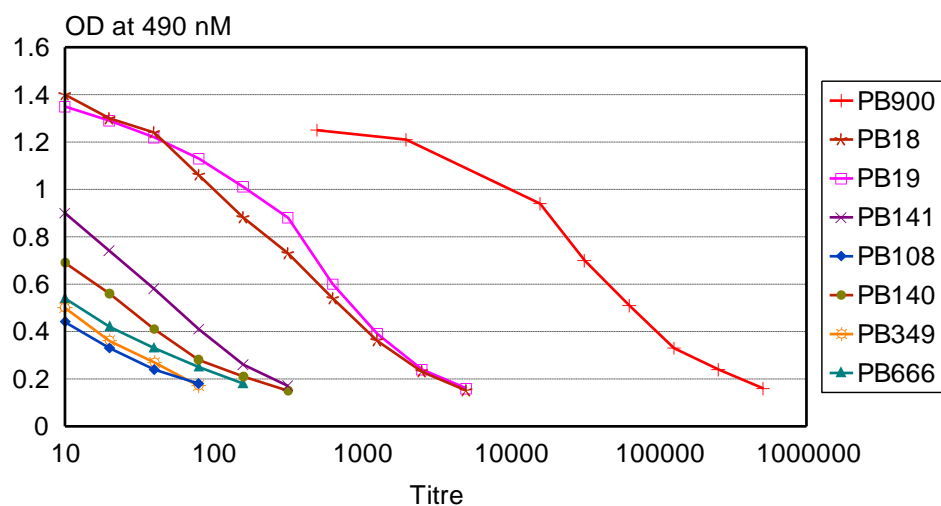
**Figure 4.13:** Anti-PS IgG graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



**Figure 4.14:** Anti-PDF IgG graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



**Figure 4.15:** Anti-PDO IgG graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



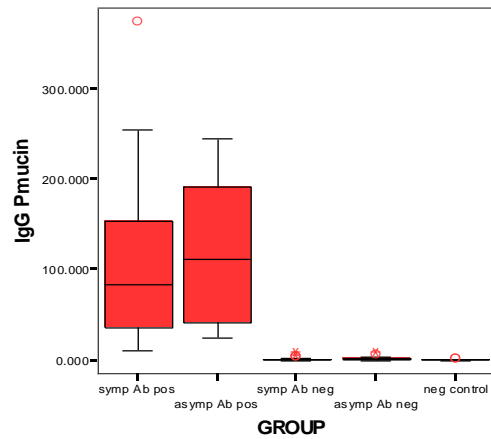
**Figure 4.16:** Anti-PIS IgG graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.

Group	Anti- mucin IgG Titres Median (range)	Anti- TM IgG Titres Median (range)	Anti- PS IgG Titres Median (range)	Anti- PDF IgG Titres Median (range)	Anti- PDO IgG Titres Median (range)	Anti- PIS IgG Titres Median (range)
<b>A</b> <b>n:25</b>	<b>84000</b> (10000-375000)	<b>291000</b> (8700-1160000)	<b>21000</b> (470-245000)	<b>134400</b> (7500-735000)	<b>145000</b> (77000-525000)	<b>56400</b> (4000-784000)
<b>B</b> <b>n:25</b>	<b>112000</b> (24000-245000)	<b>464000</b> (145500-1750000)	<b>31600</b> (470-230000)	<b>145000</b> (42000-655000)	<b>275000</b> (66000-800000)	<b>87000</b> (15000-400000)
<b>C</b> <b>n:25</b>	<b>500</b> (5-8400)	<b>1700</b> (5-50000)	<b>340</b> (5-4500)	<b>1300</b> (5-13500)	<b>1300</b> (60-36000)	<b>470</b> (5-8700)
<b>D</b> <b>n:25</b>	<b>900</b> (20-8400)	<b>1700</b> (70-12800)	<b>930</b> (20-27900)	<b>1600</b> (20-21600)	<b>2000</b> (200-77000)	<b>700</b> (20-22500)
<b>E</b> <b>n:10</b>	<b>120</b> (20-2000)	<b>300</b> (40-3500)	<b>40</b> (8-2200)	<b>300</b> (60-7500)	<b>700</b> (100-2600)	<b>100</b> (50-700)

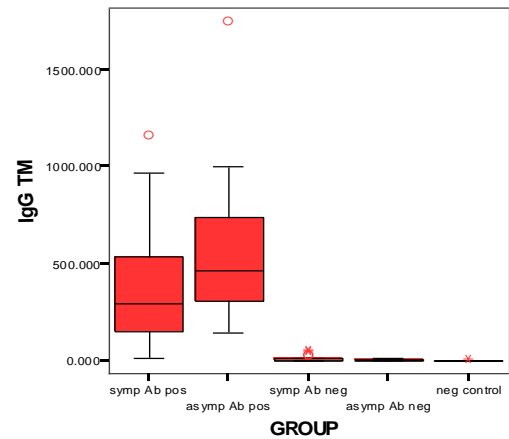
**Table 4.6:** The median and interquartile range of IgG titres against pigeon antigens in each study group; anti-PS IgG had the lowest median and range among pigeon antigens.



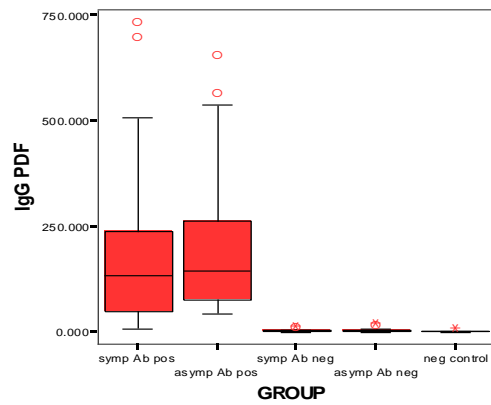
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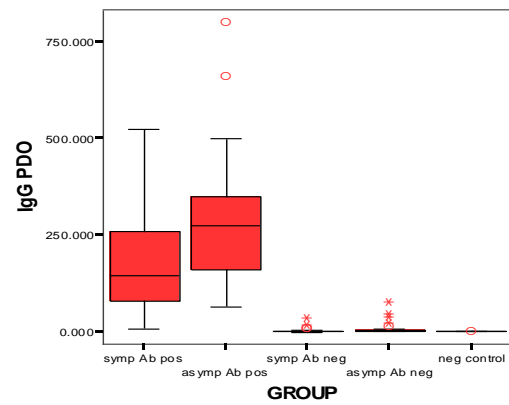
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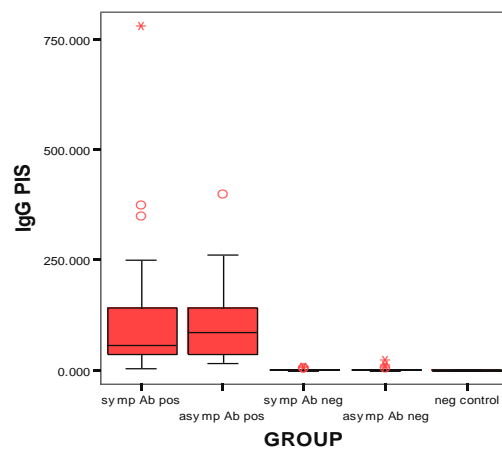
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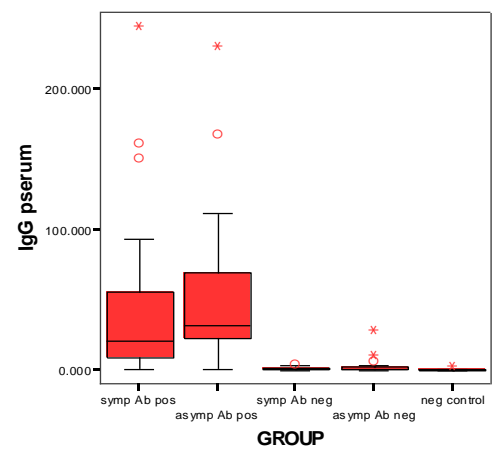
4



5



6



**Figure 4.17:** IgG titres against pigeon antigens in all study groups; 1: IgG anti- PIM, 2: IgG anti- TM, 3: IgG anti- PDF, 4: IgG anti- PDO, 5: IgG anti- PIS, 6: IgG anti- Pigeon serum.

Overall, groups A and B had significantly higher IgG titres than groups C, D and E ( $P \leq 0.001$ ). Interestingly, individuals in group B had higher IgG titres against all pigeon antigens as compared to group A (figure 4.17). Although only anti-TM and anti-PDO IgG titres were significantly higher ( $P = 0.02$  and  $P = 0.03$ , respectively).

Also the median and range of anti-TM responses were the highest followed by anti-PDO, anti-PDF, anti-PIM, anti-PIS and anti-PS responses. However, the lowest IgG titres were anti-PS.

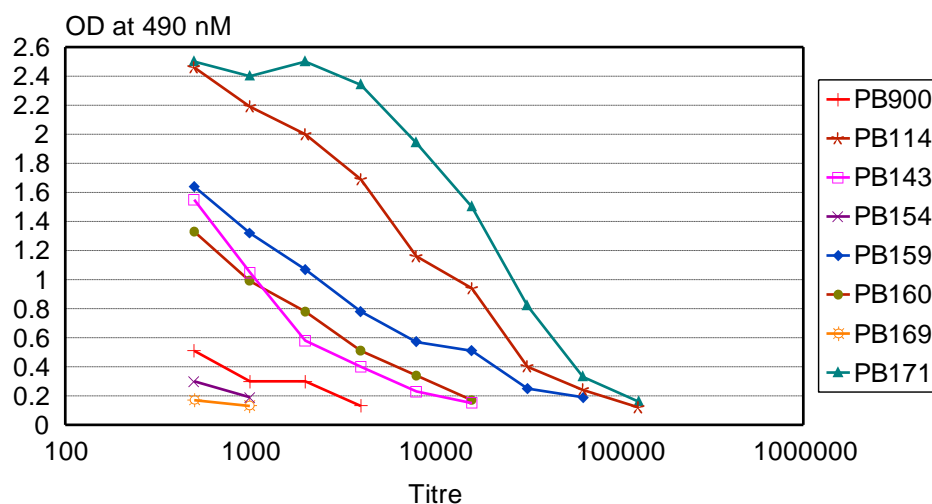
#### **4.3.6: IgG Subclass ELISA:**

ELISA was used to determine the titre of IgG1 and IgG2 against the pigeon antigens as described in section 3.10. The positive control sample was from an asymptomatic fancier with high specific antibody titres against pigeon antigens.

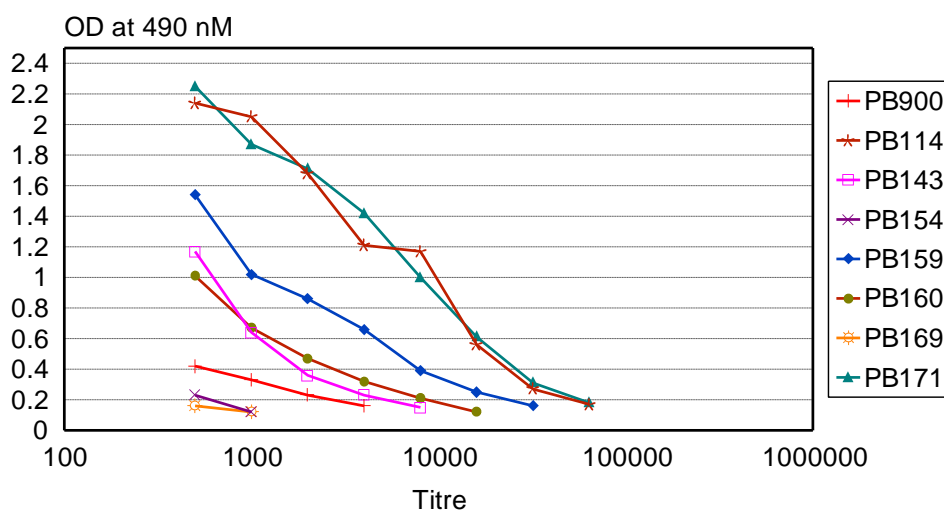
Absorbance at 490 nm was plotted against the reciprocal of the serum dilution.

The reciprocal of the dilution of each serum sample that gave an optical density of 0.2 (approximately 3 times of the background) was considered to be the end point titre of the test serum. An example of an ELISA test plate for each antigen is shown in figures 4.18-4.29.

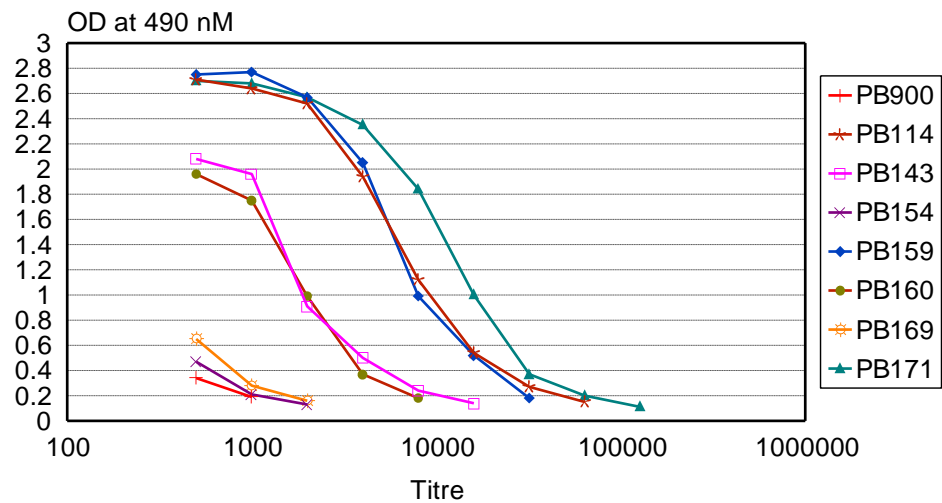
The median and the range of IgG1 and IgG2 titres for each clinical group are shown in Table 4.7 and 4.8. Also figure 4-30 and 4.31 show the level of IgG1 and IgG2 against all pigeon antigens in all study groups.



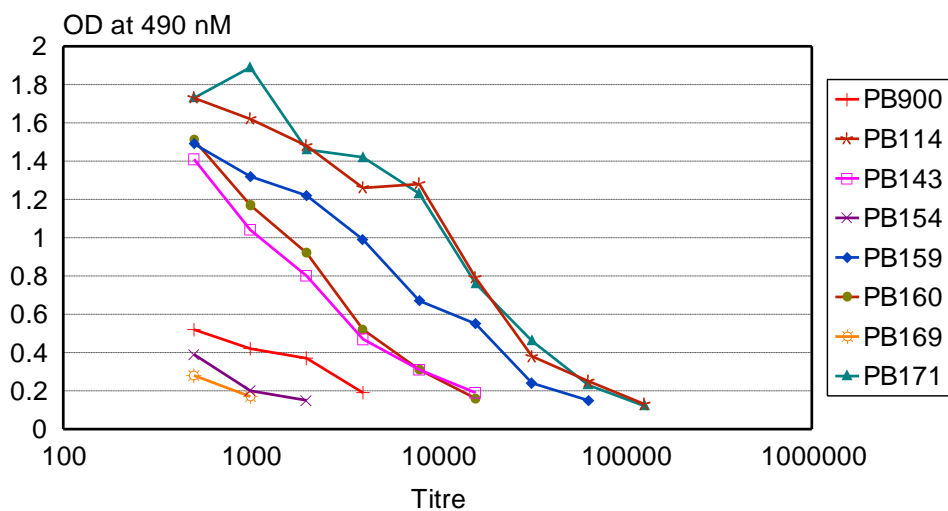
**Figure 4.18:** Anti-mucin IgG1 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



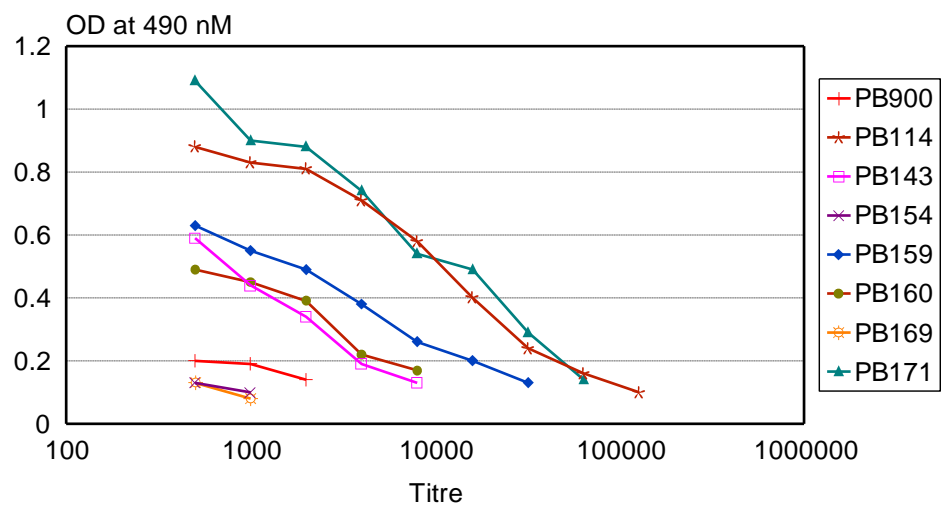
**Figure 4.19:** Anti-TM IgG1 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



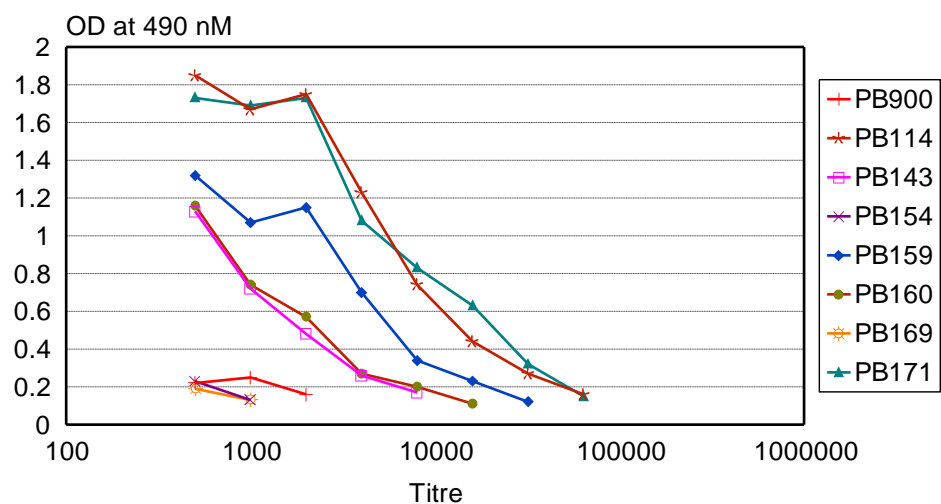
**Figure 4.20:** Anti-PS IgG1 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



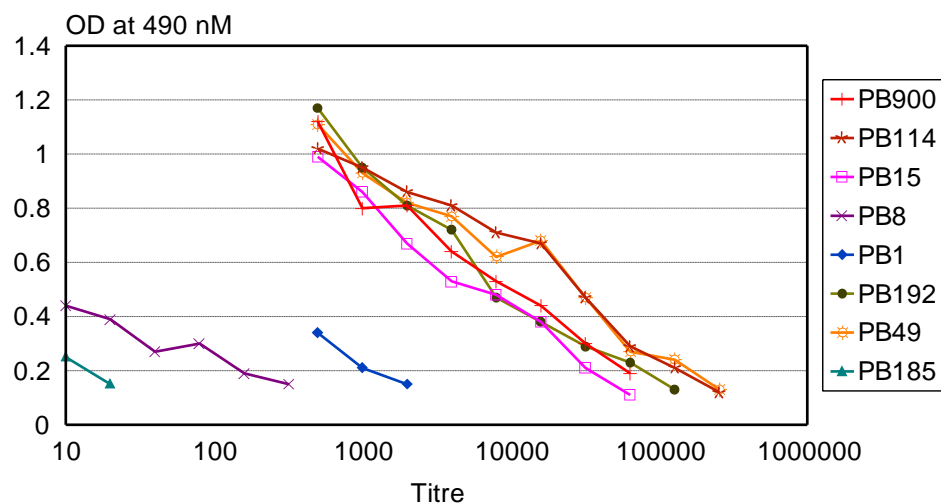
**Figure 4.21:** Anti-PDF IgG1 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



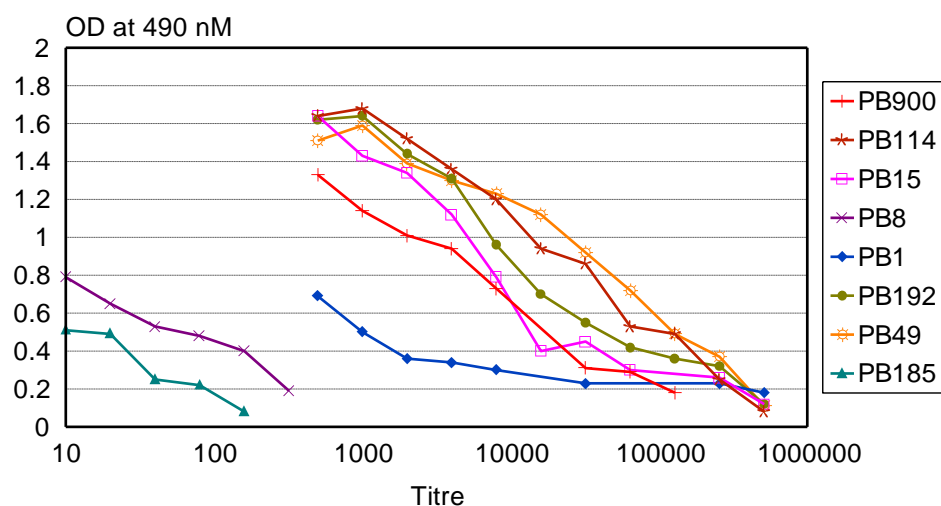
**Figure 4.22:** Anti-PDO IgG1 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



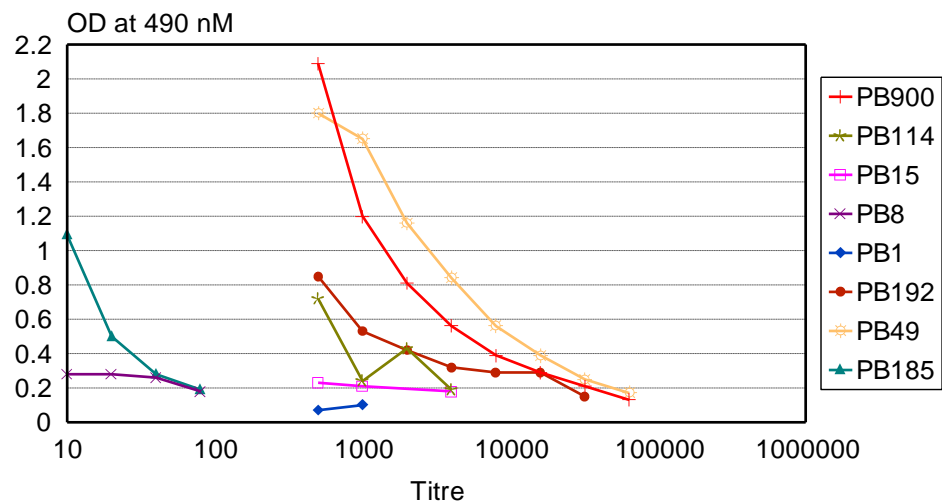
**Figure 4.23:** Anti-PIS IgG1 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



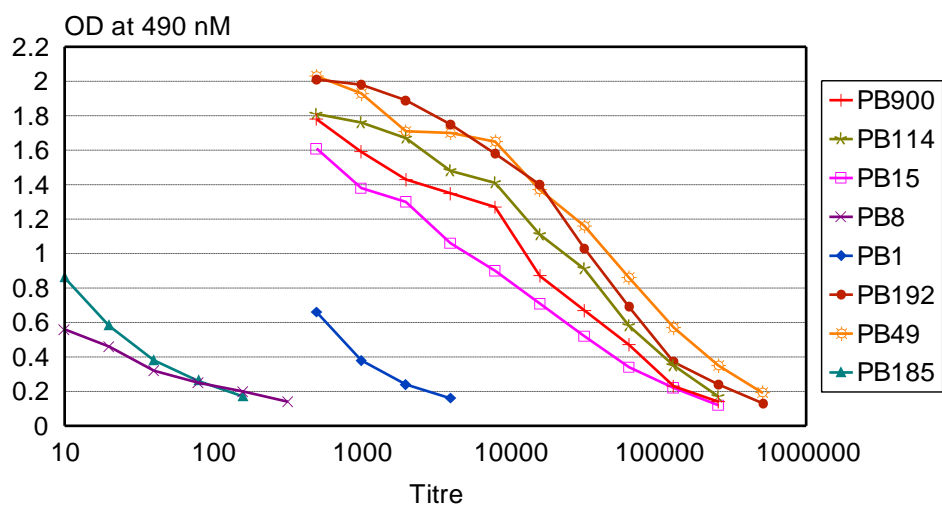
**Figure 4.24:** Anti-mucin IgG2 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



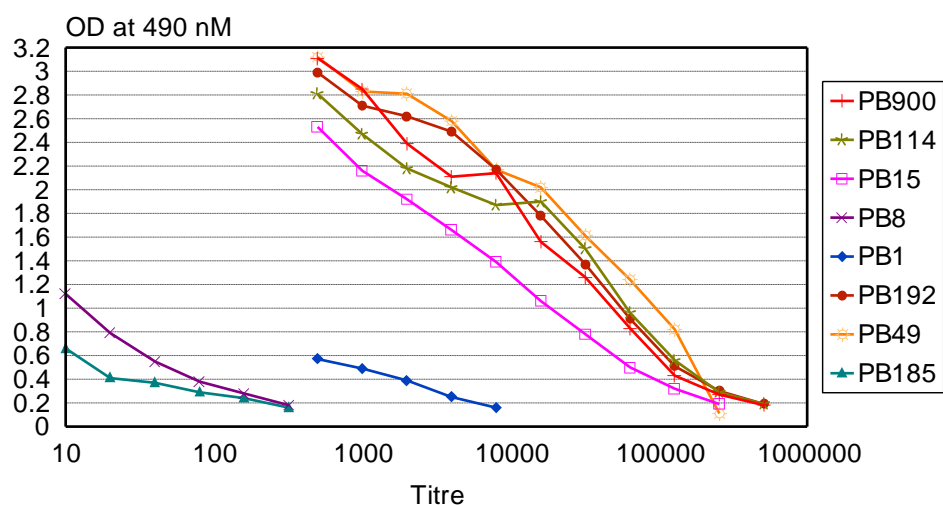
**Figure 4.25:** Anti-TM IgG2 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



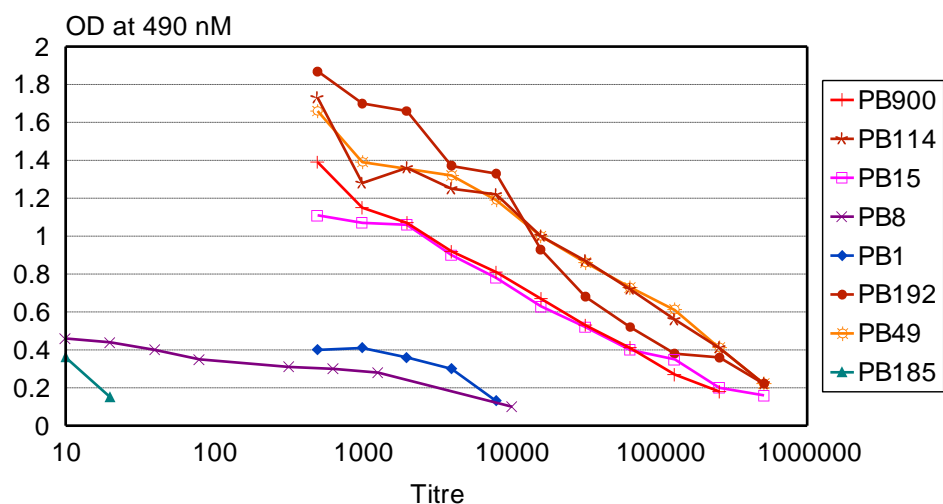
**Figure 4.26:** Anti-PS IgG2 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



**Figure 4.27:** Anti-PDF IgG2 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



**Figure 4.28:** Anti-PDO IgG2 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



**Figure 4.29:** Anti-PIS IgG2 graph of 7 pigeon fanciers' serum sample; PB900 was control positive sample. Absorbance was plotted against the reciprocal of the serum dilution and the value of each sample that gave an optical density of 0.2 was considered to be the titre of the serum.



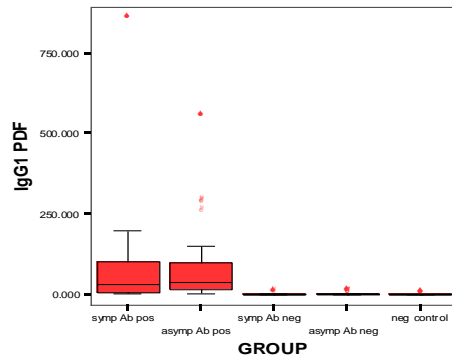
<b>Group</b>	<b>Anti- mucin IgG1 Median (range)</b>	<b>Anti- TM IgG1 Median (range)</b>	<b>Anti- PS IgG1 Median (range)</b>	<b>Anti- PDF IgG1 Median (range)</b>	<b>Anti- PDO IgG1 Median (range)</b>	<b>Anti- PIS IgG1 Median (range)</b>
<b>A n:25</b>	<b>6300</b> (100-253800)	<b>24900</b> (110-420000)	<b>11400</b> (200-243000)	<b>30000</b> (500-860000)	<b>88500</b> (4300-2160000)	<b>68000</b> (1800-1200000)
<b>B n:25</b>	<b>13600</b> (200-358700)	<b>25700</b> (500-250000)	<b>22800</b> (4100-228000)	<b>36900</b> (1700-553900)	<b>360000</b> (11800-1780000)	<b>72000</b> (5700-952000)
<b>C n:25</b>	<b>30</b> (7-1000)	<b>150</b> (6-5000)	<b>30</b> (4-5.1000)	<b>50</b> (9-4400)	<b>2100</b> (30-17800)	<b>100</b> (6-12300)
<b>D n:25</b>	<b>350</b> (2-2800)	<b>400</b> (10-3700)	<b>300</b> (5-5700)	<b>200</b> (5-6900)	<b>170</b> (100-64200)	<b>400</b> (6-33600)
<b>E n:10</b>	<b>30</b> (7-400)	<b>70</b> (10-300)	<b>30</b> (4-200)	<b>20</b> (9-700)	<b>200</b> (10-1300)	<b>40</b> (6-800)

**Table 4.7:** The median and interquartile range of IgG1 titres against pigeon antigens in each study group.

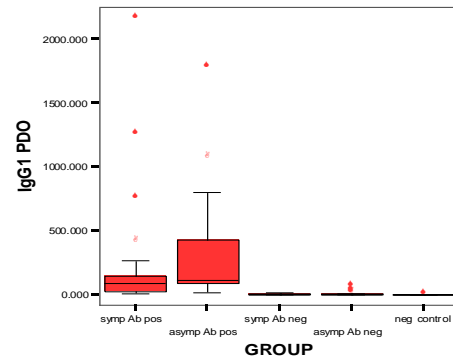
Group	Anti- mucin IgG2 Median (range)	Anti- TM IgG2 Median (range)	Anti- PS IgG2 Median (range)	Anti- PDF IgG2 Median (range)	Anti- PDO IgG2 Median (range)	Anti- PIS IgG2 Median (range)
<b>A</b> <b>n:25</b>	<b>22500</b> (500-275000)	<b>66000</b> (300-375000)	<b>7300</b> (100-49200)	<b>150000</b> (200-500000)	<b>199000</b> (200-800000)	<b>124500</b> (200-625000)
<b>B</b> <b>n:25</b>	<b>38500</b> (5800-242000)	<b>66000</b> (4600-438000)	<b>11200</b> (500-294000)	<b>249000</b> (99600-780000)	<b>226000</b> (93100-1120000)	<b>300000</b> (56200-625000)
<b>C</b> <b>n:25</b>	<b>100</b> (3-10000)	<b>100</b> (5-9900)	<b>100</b> (3-11700)	<b>900</b> (4-25000)	<b>1600</b> (2-66500)	<b>300</b> (4-31200)
<b>D</b> <b>n:25</b>	<b>300</b> (0.02-5.8)	<b>300</b> (0.02-219)	<b>200</b> (0.01-4.2)	<b>900</b> (0.04-75)	<b>200</b> (0.1-53.2)	<b>2400</b> (0.02-156)
<b>E</b> <b>n:10</b>	<b>100</b> (0.001-2)	<b>35</b> (0.005-3.7)	<b>30</b> (0.01-0.2)	<b>200</b> (0.05-3.8)	<b>400</b> (0.02-2.4)	<b>100</b> (0.03-3.2)

**Table 4.8:** The median and interquartile range of IgG2 titres against pigeon antigens in each study group.

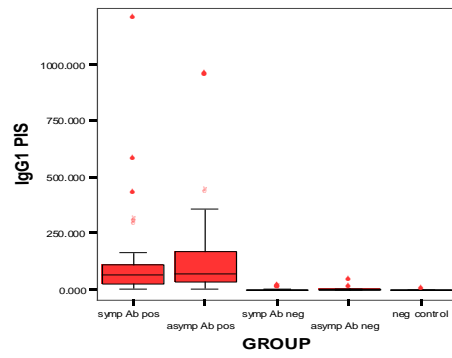
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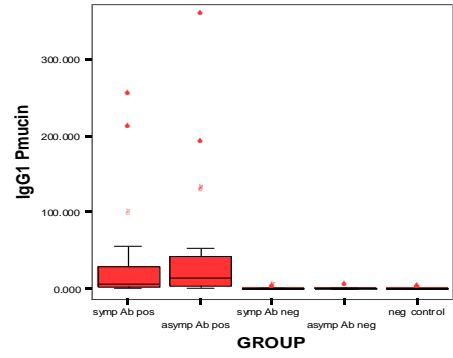
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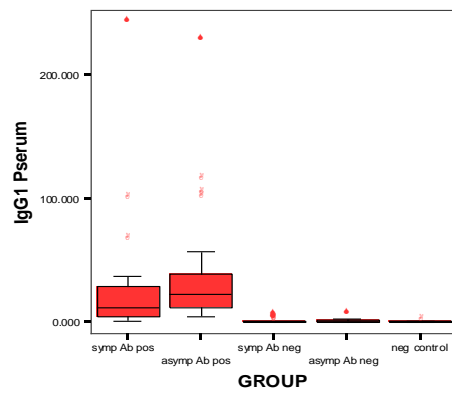
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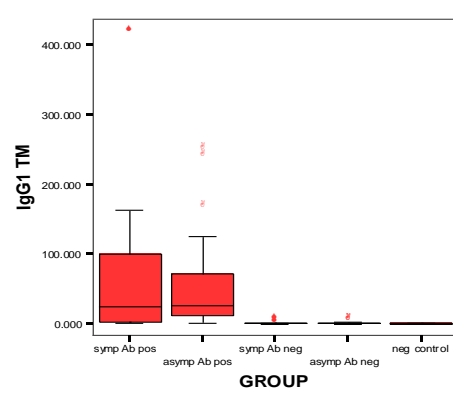
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E

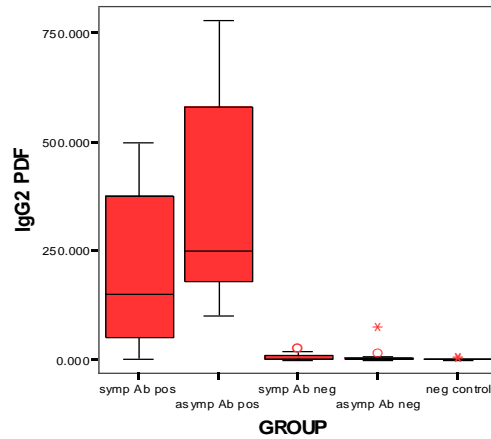


F

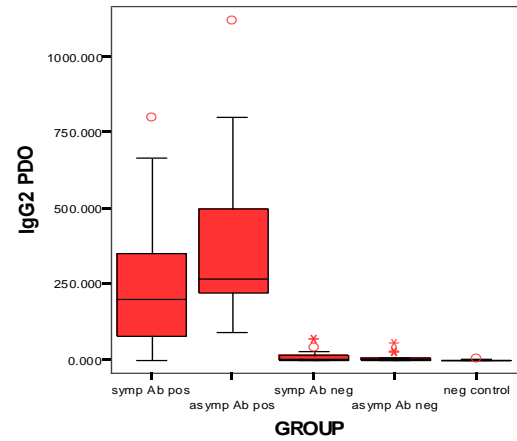


**Figure 4.30:** IgG1 levels against pigeon antigens in all study groups; A: IgG1 anti- PIM, B: IgG1 anti- TM, C: IgG1 anti- PDF, D: IgG1 anti- PDO, E: IgG1 anti- PIS, F: IgG1 anti- Pigeon serum.

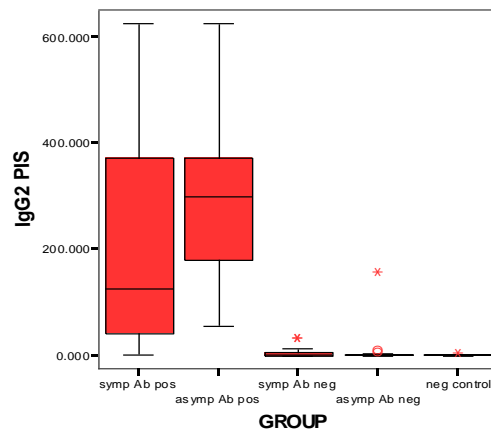
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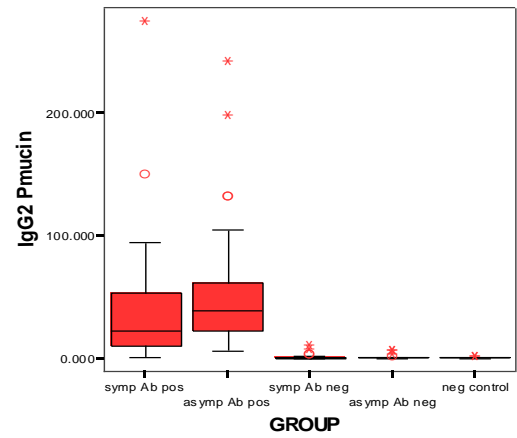
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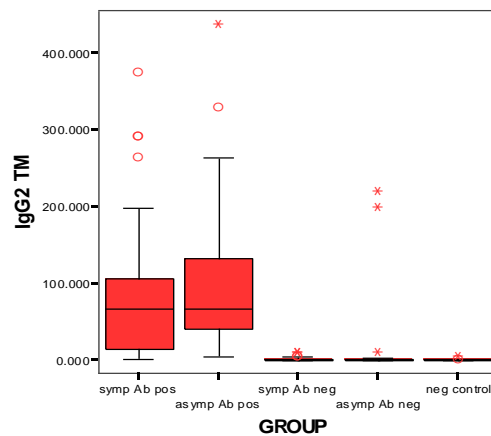
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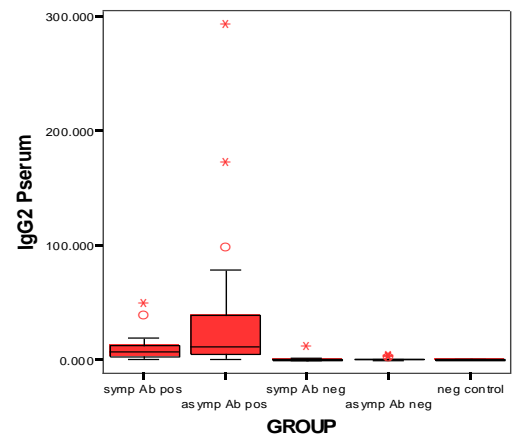
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6



**Figure 4.31:** IgG2 titres against pigeon antigens in all study groups; 1: IgG2 anti- PDF, 2: IgG2 anti- PDO, 3: IgG2 anti- PIS, 4: IgG2 anti- PIM, 5: IgG2 anti- TM, 6: IgG2 anti- Pigeon serum.

It should be emphasized that the comparison between antibodies titres were made within the IgG subclasses of the group of pigeon fanciers and not between different subclass responses due to differences in the sensitivity of the IgG subclass ELISAs.

Firstly the median and range of IgG1 titres against PIM, TM, PDO, PDF and PIS was higher than the IgG1 titres against PS in both group A and B individuals. This may suggest that either those antigens are more effective in B cell stimulation and antibody production than PS or it may be that individuals are exposed to higher concentration of those antigens thus triggering higher responses. Groups A and B also had significantly higher titres of IgG1 against pigeon antigens compared to groups C, D and E ( $P \leq 0.001$ ). However, there were no significant differences between IgG1 titres against pigeon antigens in groups C and D except for IgG1 anti-mucin ( $P=0.02$ ). IgG1 titres against all pigeon antigens in group B were higher than group A, but it was only significant for anti-PS IgG1 ( $P=0.04$ ).

Secondly the median and range of IgG2 titres against PIM, TM, PDO, PDF and PIS was also higher than the IgG2 titres against PS in both group A and B individuals. Groups A and B also had significantly higher titres of IgG2 against pigeon antigens compared to groups C, D and E ( $P \leq 0.001$ ). However, there were no significant differences between IgG2 titres against pigeon antigens in groups C and D. Also the IgG2 titres against all pigeon antigens in group B were higher than group A. These differences were significant for anti-PDF IgG2 ( $P=0.02$ ), anti-PDO IgG2 ( $P=0.04$ ) and anti-PIS IgG2 ( $P=0.03$ ). IgG2 titres against PS had the lowest median and range compared to other pigeon antigens suggesting the presence of high carbohydrate content of these antigens, since IgG2 is associated with responses to polysaccharide antigens (Hammarstrom & Smith, 1986; Obukhanych & Nussenzweig, 2006).

Finally the antibody titres (IgG, IgG1 and IgG2) against all pigeon antigens were compared with the smoking status of pigeon fanciers by Multiple Regression test.

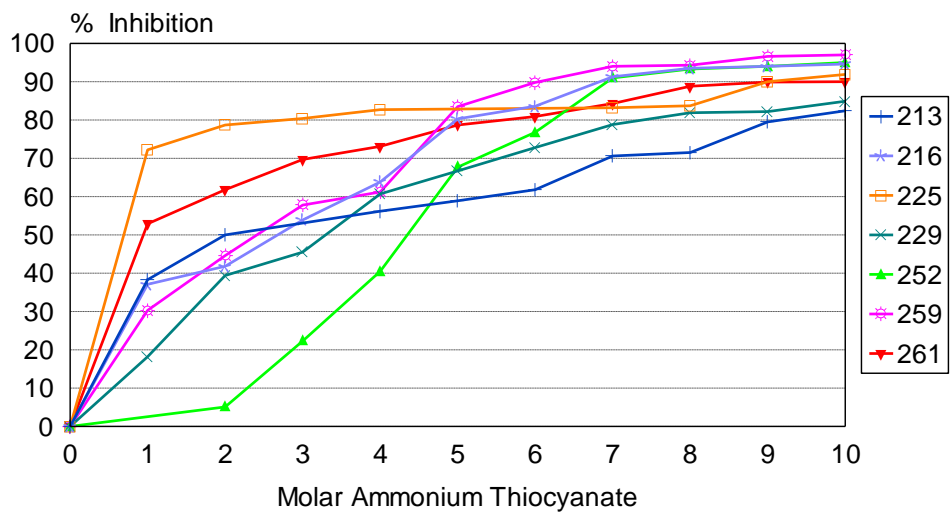
There was a significant association between antibody titres and smoking status, with those fanciers who smoked having significantly lower antibody titres than non-smokers ( $P < 0.05$ ).

#### **4.3.7: Inhibition ELISA to Measure Antibody Avidity:**

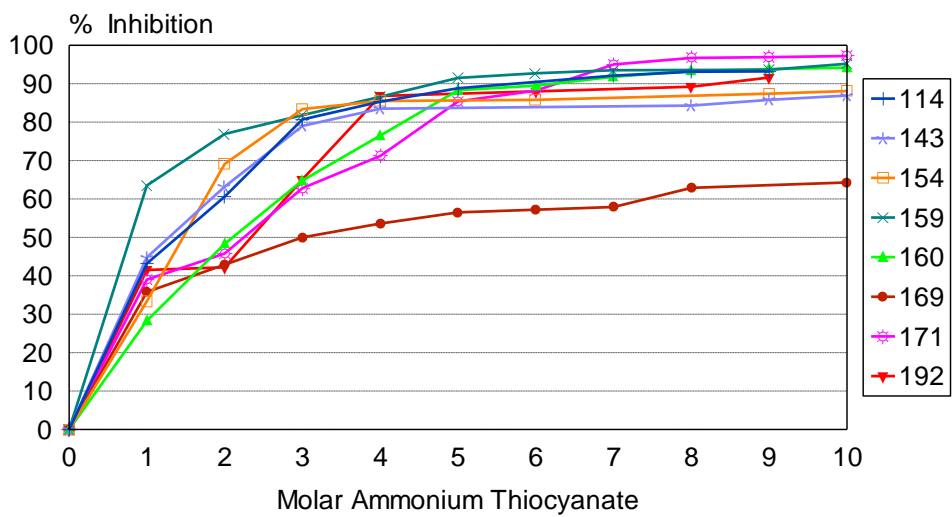
Antibody avidity is an important aspect of a protective immune response and can be assessed by chaotropic or inhibition ELISA. This technique utilizes a chaotropic reagent such as ammonium thiocyanate which disrupts antigen-antibody binding by interfering with the electrostatic, hydrophobic bonds in immune complexes. It also chelates the proton of the hydrogen bonds which stabilize immune complexes (Macdonald, Hosking & Jones, 1988; Giovannoni, Chapman & Thompson, 2006). The avidity index (AI) is determined as the concentration of ammonium thiocyanate that inhibits antibody binding by 50% in an ELISA (Richmond *et al.*, 2001). Thus the higher avidity of an antigen-antibody complex the higher the concentration of chaotropic reagent needed to disrupt the complex.

This experiment was carried out as previously described in section 3.11 and AI was defined as the concentration of ammonium thiocyanate required to reduce the OD by 50% in comparison to sample without chaotropic reagent. This is determined from a graph plotting the percentage inhibition against the concentration of ammonium thiocyanate. Individual data for each IgG subclass tested the six pigeon antigens ie presented in figures 4.32- 4.43. The mean and range of AI for IgG1 and IgG2 against pigeon antigens are shown in Table 4.9 and 4.10. Figure 4.44 shows AI levels for IgG1 and IgG2 against PDO and PDF in group A and B.

Considering these data, group A generally had a higher mean and range of AI for IgG1 against pigeon antigens as compared to group B, however, the differences were only significant For IgG1 against PDO ( $P=0.006$ ). Group A also had higher AI for IgG2 against 5 antigens, with significant differences being seen for IgG2 against PDO and PDF ( $P=0.002$  and  $P\leq 0.001$ , respectively) as shown in figure 4.44. This data showed that higher AI for IgG1 and IgG2 could be used as a diagnostic tool. The AI for IgG2 against mucin was higher in group B than group A but it was not significant ( $P>0.05$ ). Interestingly, the mean and range of AI for IgG2 were higher than IgG1 in both groups A and B which may suggest the presence of more IgG2 in the immune complexes.

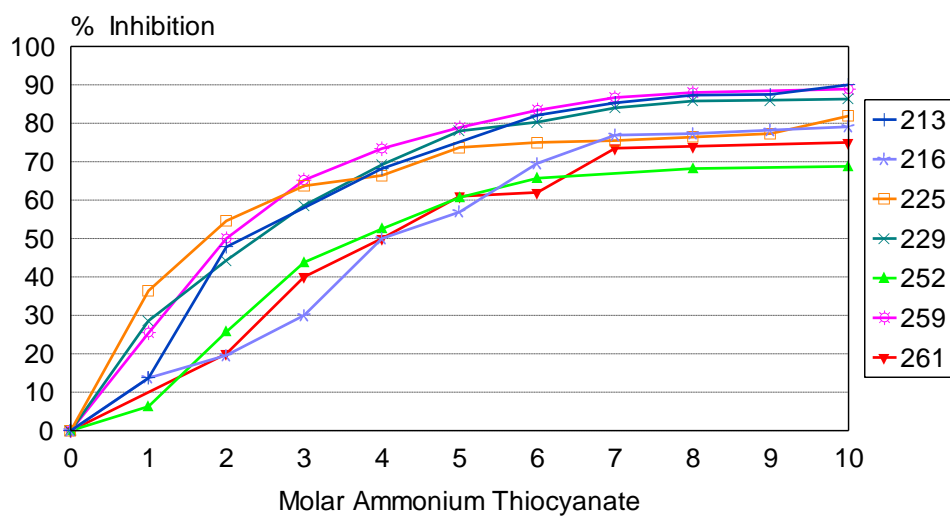


**Figure 4.32:** Inhibition ELISA for IgG1 against mucin; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.

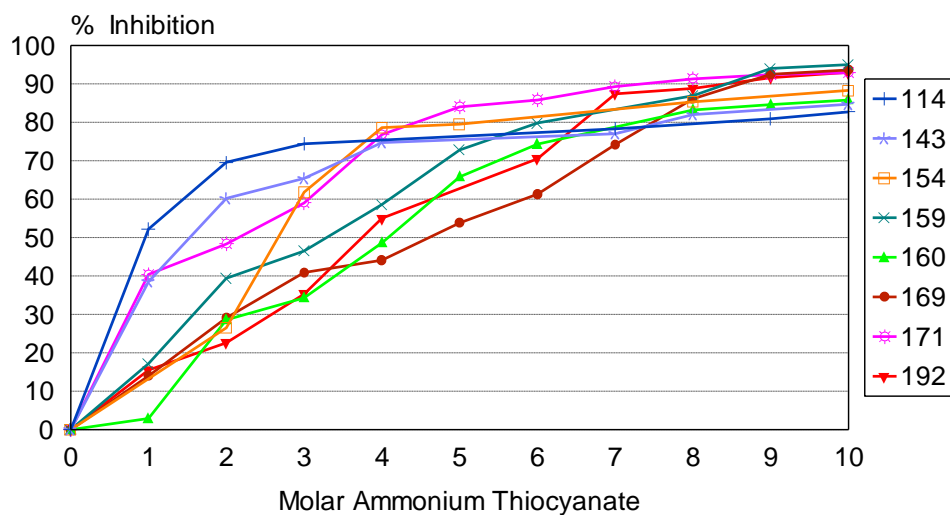


**Figure 4.33:** Inhibition ELISA for IgG1 against TM; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.

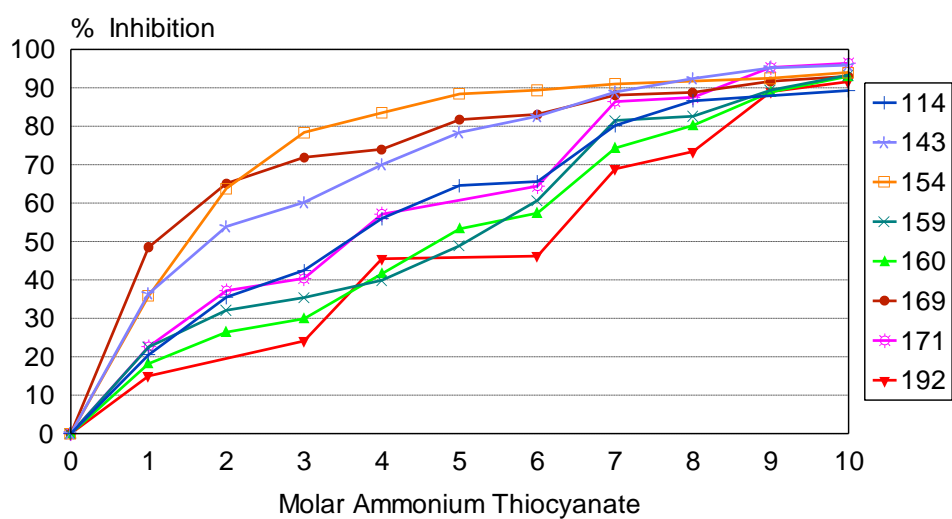




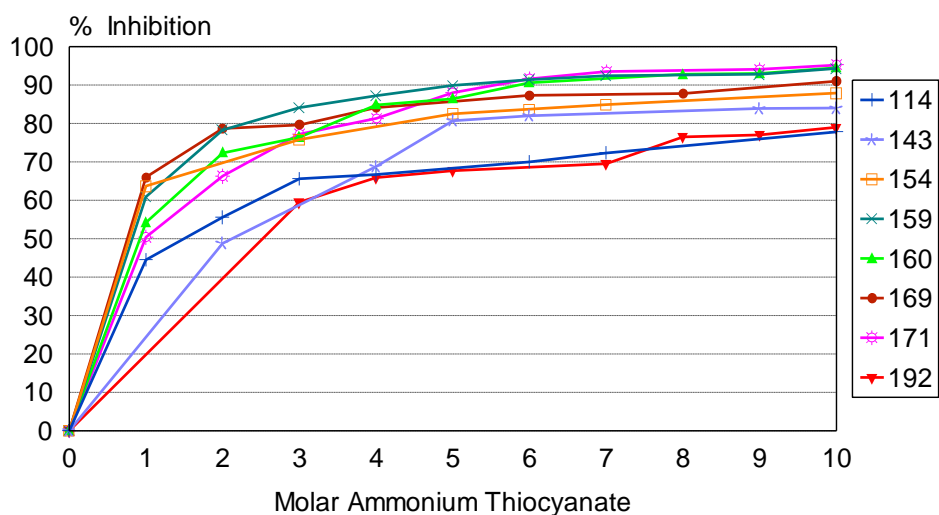
**Figure 4.34:** Inhibition ELISA for IgG1 against PS; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



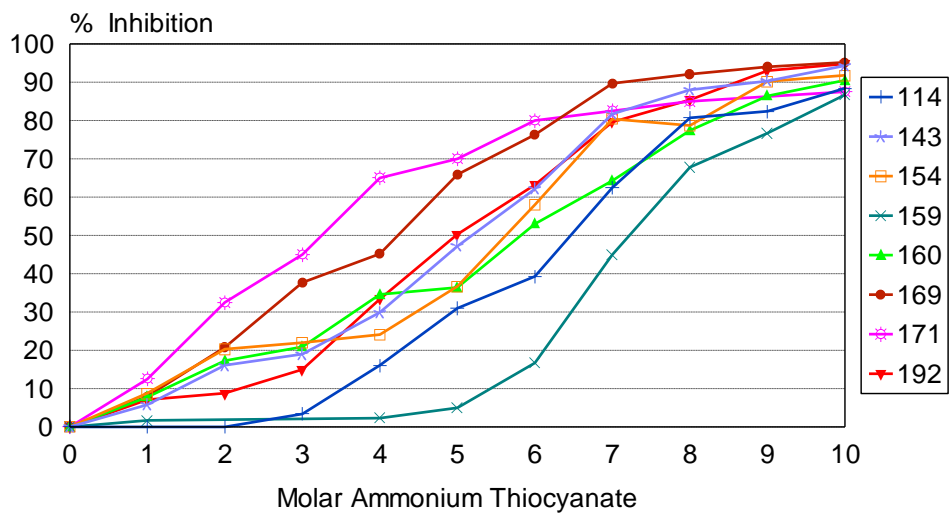
**Figure 4.35:** Inhibition ELISA for IgG1 against PDF; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



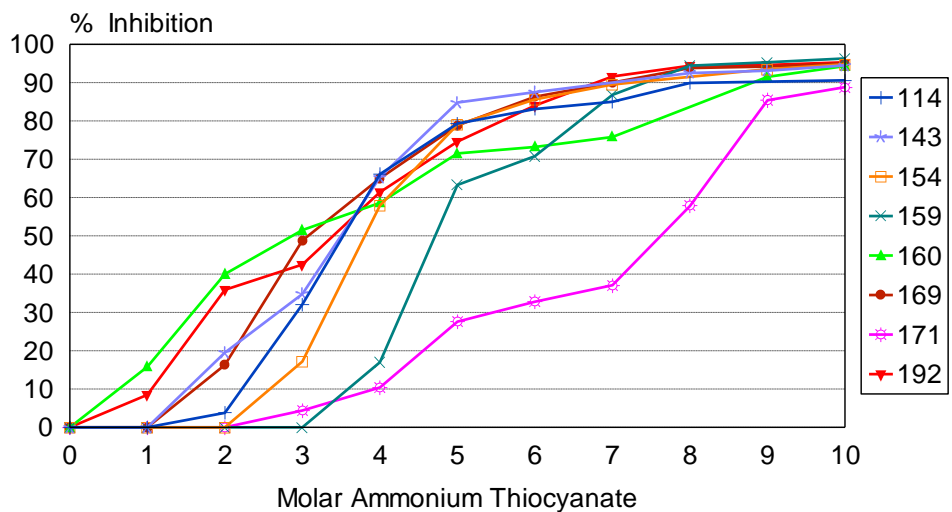
**Figure 3.36:** Inhibition ELISA for IgG1 against PDO; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



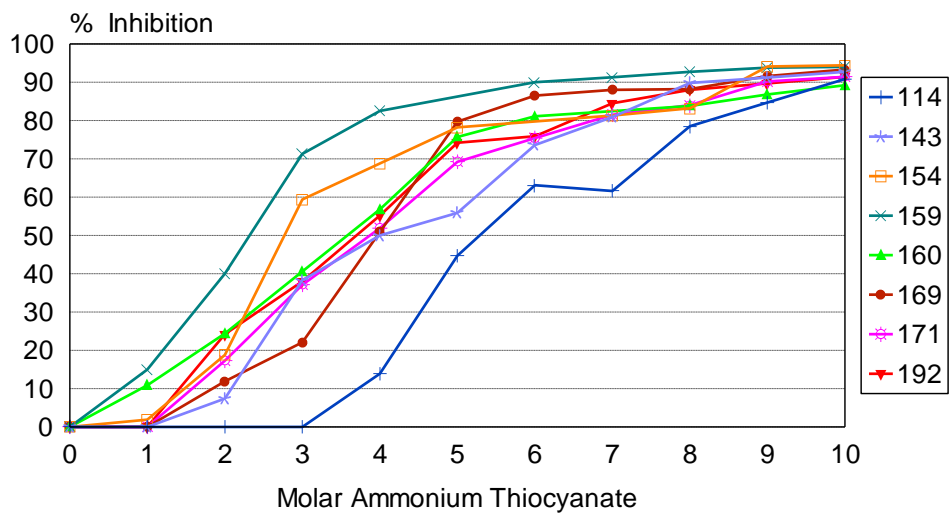
**Figure 3.37:** Inhibition ELISA for IgG1 against PIS; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



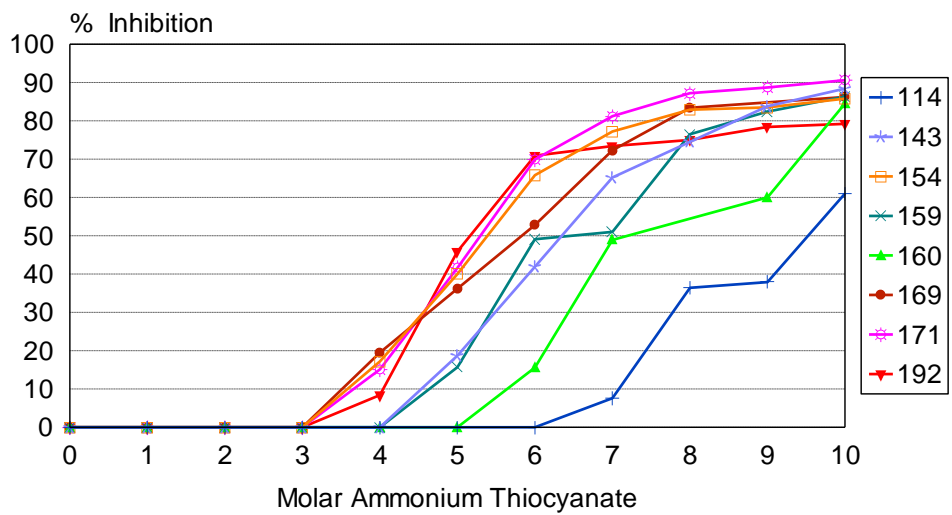
**Figure 3.38:** Inhibition ELISA for IgG2 against mucin; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



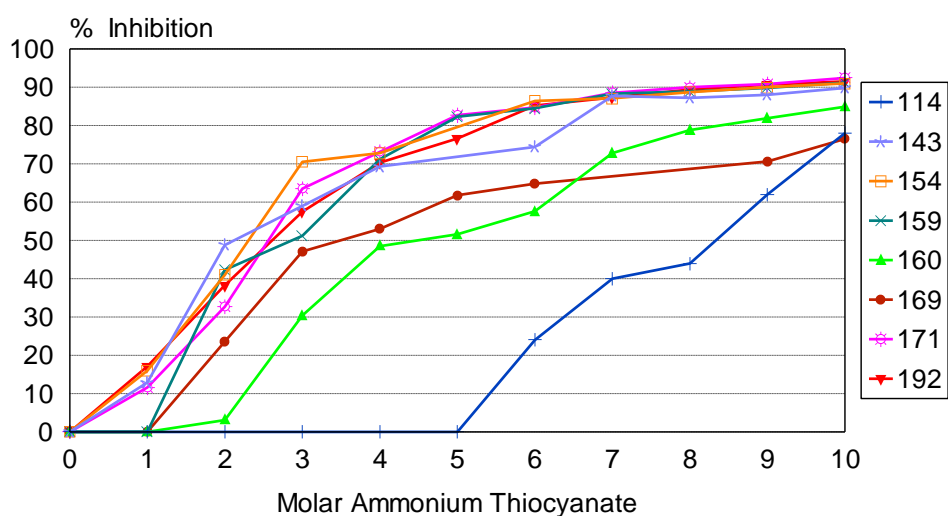
**Figure 3.39:** Inhibition ELISA for IgG2 against TM; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



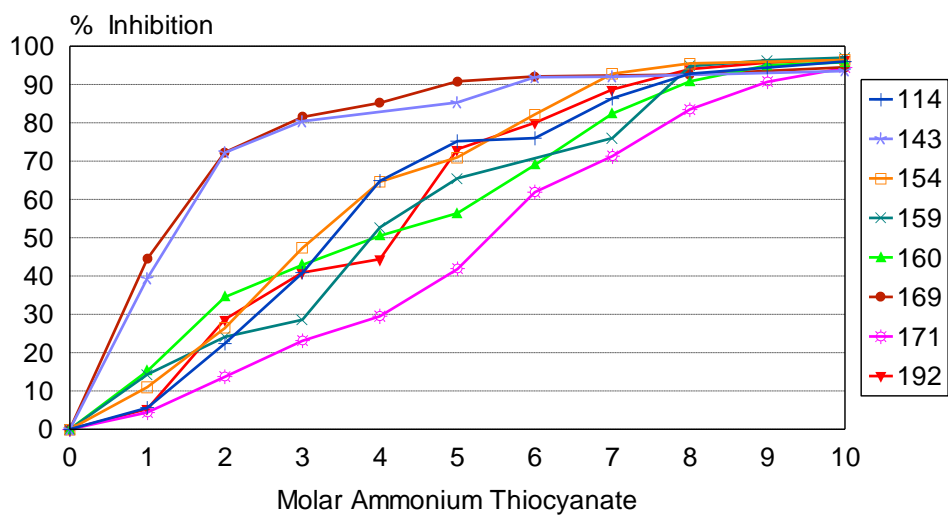
**Figure 4.40:** Inhibition ELISA for IgG2 against PS; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



**Figure 4.41:** Inhibition ELISA for IgG2 against PDF; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



**Figure 4.42:** Inhibition ELISA for IgG2 against PDO; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.



**Figure 4.43:** Inhibition ELISA for IgG2 against PIS; percentage inhibition was plotted against the concentration of ammonium thiocyanate. AI is defined as the concentration of ammonium thiocyanate that reduces the OD by 50%.

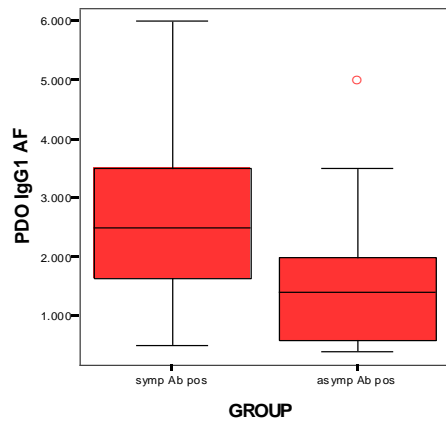
Groups	Mucin	TM	PS	PDF	PDO	PIS
<b>Group A</b> <b>(n=25)</b>						
*Mean	<b>2.21</b>	<b>1.72</b>	<b>2.31</b>	<b>2.76</b>	<b>2.69</b>	<b>1.21</b>
*Range	(0.6-5)	(0.6-4.2)	(0.8-4)	(0.6-5.5)	(0.5-6)	(0.6-2.5)
*SD	<b>1.29</b>	<b>0.95</b>	<b>1.10</b>	<b>1.45</b>	<b>1.52</b>	<b>0.58</b>
<b>Group B</b> <b>(n=25)</b>						
*Mean	<b>1.59</b>	<b>1.27</b>	<b>2.15</b>	<b>2.61</b>	<b>1.59</b>	<b>1.06</b>
*Range	(0.5-4)	(0.5-3.5)	(0.6-5.4)	(1-4.5)	(0.4-5)	(0.6-3)
*SD	<b>0.9</b>	<b>0.7</b>	<b>1.19</b>	<b>0.95</b>	<b>1.14</b>	<b>0.52</b>

**Table 4.9:** The mean, range and standard deviation (SD) of avidity index (AI) of IgG1 against pigeon antigens in group A and B (95% Confidence Interval); Group A had higher AI for IgG1 against all pigeon antigens than group B.

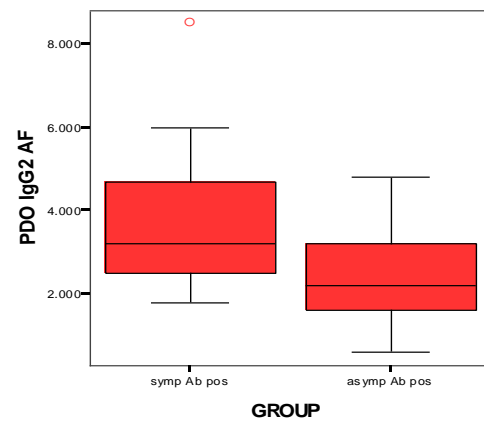
<b>Groups</b>	<b>Mucin</b>	<b>TM</b>	<b>PS</b>	<b>PDF</b>	<b>PDO</b>	<b>PIS</b>
<b>Group A</b> <b>(n=25)</b>						
*Mean	<b>4.51</b>	<b>3.57</b>	<b>3.59</b>	<b>6.12</b>	<b>3.66</b>	<b>2.67</b>
*Range	(1.2-7.5)	(1.5-7.5)	(0.6-7.5)	(2.2-10)	(1.8-8.5)	(1.2-5.5)
*SD	<b>1.6</b>	<b>1.34</b>	<b>1.61</b>	<b>1.63</b>	<b>1.6</b>	<b>1.26</b>
<b>Group B</b> <b>(n=25)</b>						
*Mean	<b>5.12</b>	<b>3.24</b>	<b>3.22</b>	<b>4.05</b>	<b>2.39</b>	<b>2.09</b>
*Range	(2-8)	(0.5-6)	(1-7)	(0.7-8.5)	(0.6-4.8)	(0.5-4.6)
*SD	<b>1.5</b>	<b>1.29</b>	<b>1.54</b>	<b>1.82</b>	<b>0.94</b>	<b>0.96</b>

**Table 4.10:** The mean, range and standard deviation (SD) of avidity index (AI) of IgG2 against pigeon antigens in group A and B (95% Confidence Interval); Group A had higher AI for IgG2 against all pigeon antigens except mucin compared to group B.

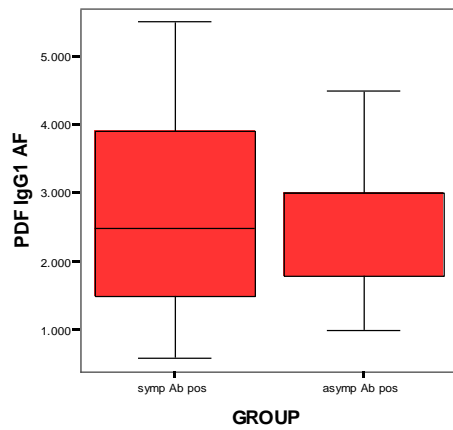
1



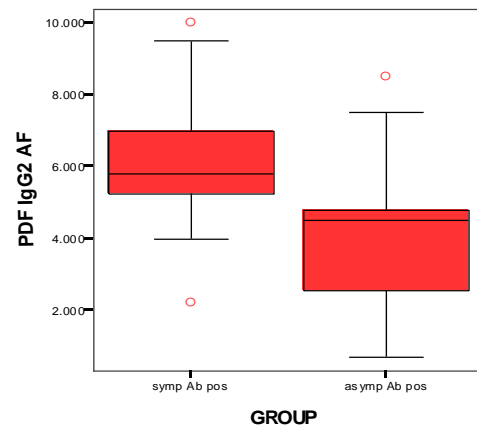
2



3



4



**Figure 4.44:** AI levels for anti- PDO IgG1 (1), anti- PDO IgG2 (2), anti- PDF IgG1 (3) and anti-PDF IgG2 (4) in group A and B; Group A had much higher AI of IgG2 against both antigens and higher AI of IgG1 against PDO.



#### **4.3.8: Microcalorimetry:**

In order to achieve a better understanding of the interaction between antigen and antibody (avidity of the antibody), the thermodynamics of the antigen-antibody reaction were measured by microcalorimetry. In this series of experiments an injection syringe (pipette) containing a ligand (antibody) was titrated against a binding partner or macro molecule (antigen) at a constant temperature. The temperature difference between the reference cell and the sample cell was measured by VP-ITC (MicroCal LLC, Milton Keynes, UK) (section 3.12).

The heat generated by antigen-antibody interactions ( $\Delta H$ ) following each injection was measured by the system. The signal diminishes as the antigen binding sites in the sample cell became saturated with added ligand. Differential power (DP) in  $\mu\text{cal/sec}$  is the difference in power between the sample cell and the reference cell. A positive DP value means the reference cell is hotter than the sample cell. Thus, an endothermic reaction needs more feedback heat to maintain the same temperature, resulting in a positive deflection in the DP values. On the other hand, a negative DP value means the sample cell is hotter than the reference cell. In an exothermic reaction the feed back heat provided to the sample cell to maintain the temperature equilibrium between the sample and the reference cell is decreasing, which causes the DP to deflect in a negative direction.

A titration curve is obtained when the heat from each injection is plotted against the ratio of antigen and antibody in the reaction cell.  $\Delta H$  can then be extracted from the curve (Wiseman *et al.*, 1989; Bundle & Sigurskjold, 1994).

PDO was used as the tested antigen in this experiment since it was shown that fanciers in group A have a significant higher AI for IgG1 and IgG2 against PDO in comparison with group B ( $P=0.006$  and  $P=0.002$ , respectively). To determine the optimal concentration of ligand and macromolecule, in order to have a sigmoid slope in the final graph, one sample

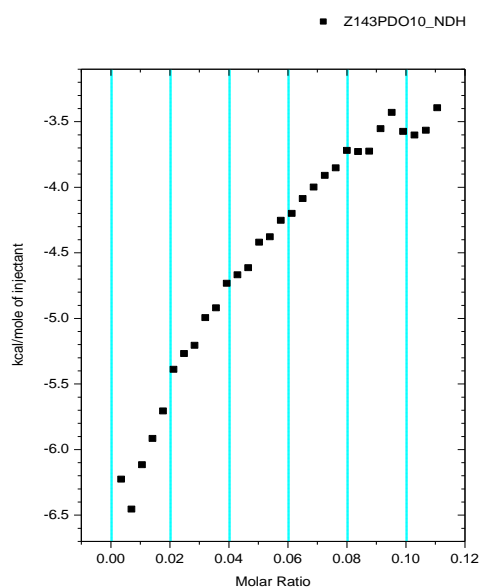
from group A and B diluted 1/2 and 1/10 into PBS and were tested against PDO at concentrations of 1 mg/ml and 2 mg/ml. The results are shown in figures 4.45 and 4.46. The best sigmoidal slopes were achieved with both samples at a 1/2 dilution with antigen concentration of 2 mg/ml.

10 serum samples from group A and 11 from group B were selected for this series of experiments. The aim was to choose the samples with almost similar anti-PDO IgG titres in order to minimise any effects of antibody concentration that may be seen between samples. Thus any differences in  $\Delta H$  detected would be as a result of differences in avidity/affinity of the antibody in the serum sample and not concentration.

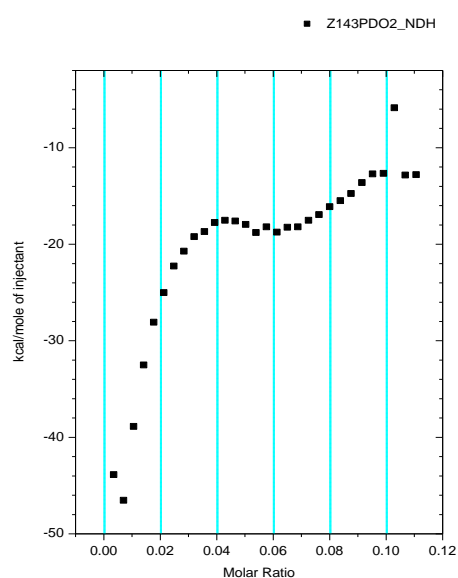
The group A and group B serum samples are shown in table 4.11 with the IgG titres. Results for the samples with similar antibody titres to PDO were compared.

Each serum sample was tested against PDO and PBS as a control. The final graph ( $\Delta H$ ) was the result of subtracting the control graph from the main graph since the interaction between the ligand and the buffer (PBS) produces some heat. Figures 4.47 and 4.48 show this process for sample 143 (group A) and sample 37 (group B). The other graphs are not shown.

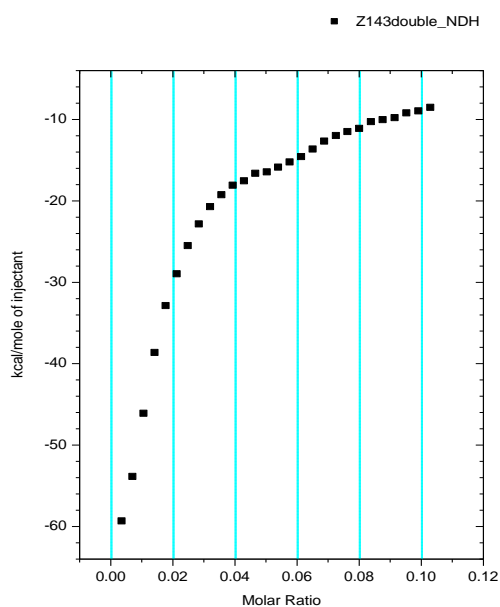
1)



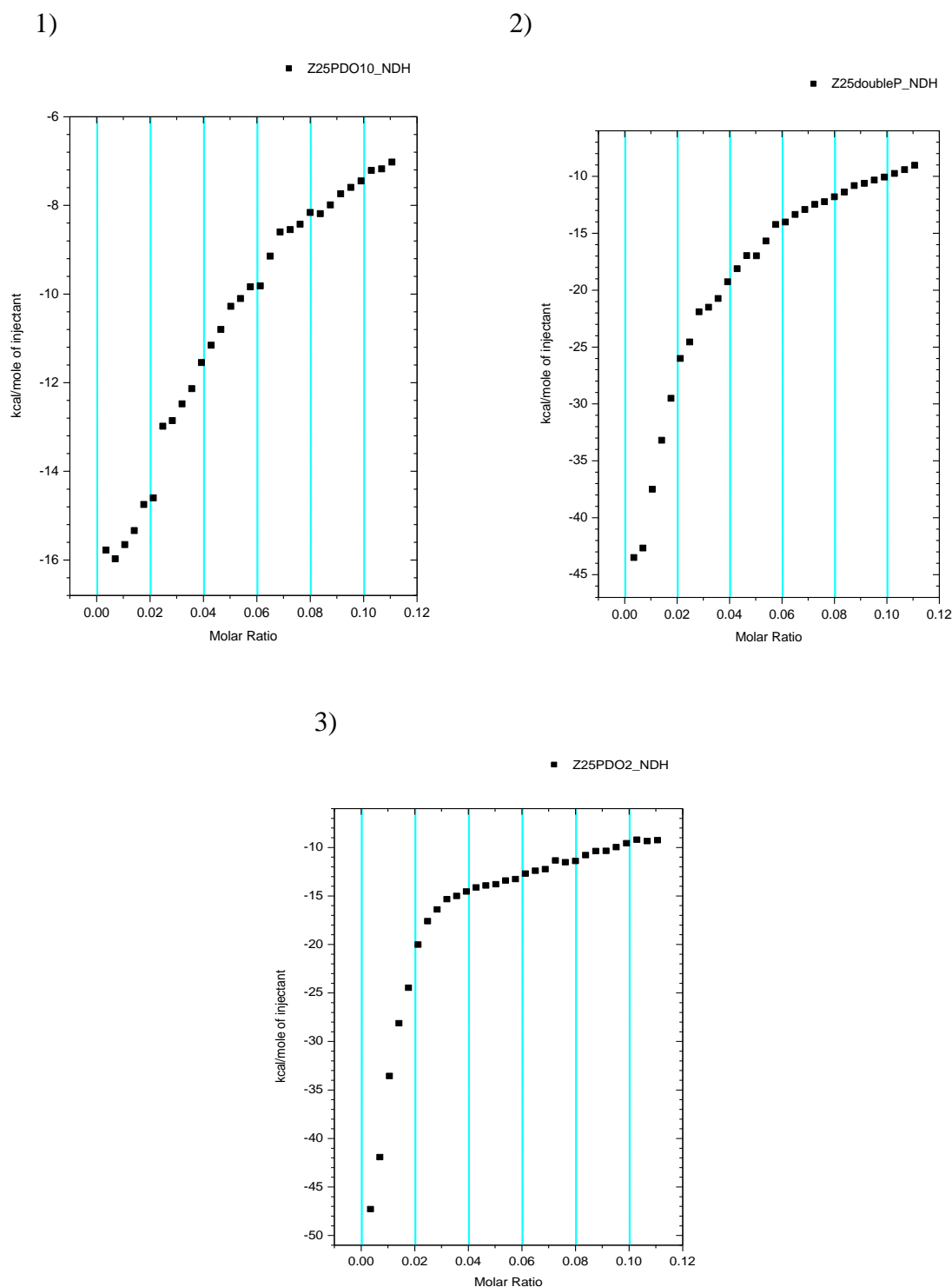
2)



3)



**Figure 4.45:** Initial ITC experiments for sample 143 (group A) in order to find the optimal concentration of the ligand and macromolecule; the results show that a 1/2 dilution of serum (ligand) and PDO at 2mg/ml (macromolecule) gives the best sigmoidal slope. 1) 1/10 diluted serum into PBS and PDO at 1 mg/ml; 2) 1/2 diluted serum into PBS and PDO at 1 mg/ml; 3) 1/2 diluted serum into PBS and PDO at 2 mg/ml.

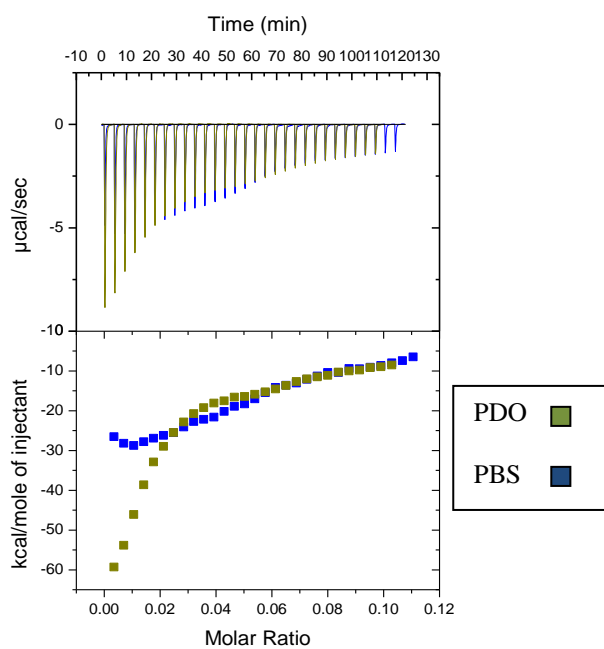


**Figure 4.46:** Initial ITC experiments for sample 25 (group B) in order to find the optimal concentration of the ligand and macromolecule; the results show that a 1/2 dilution of serum (ligand) and PDO at 2mg/ml (macromolecule) gives the best sigmoidal slope. 1) 1/10 diluted serum into PBS and PDO at 1 mg/ml; 2) 1/2diluted serum into PBS and PDO at 1 mg/ml; 3) 1/2diluted serum into PBS and PDO at 2 mg/ml.

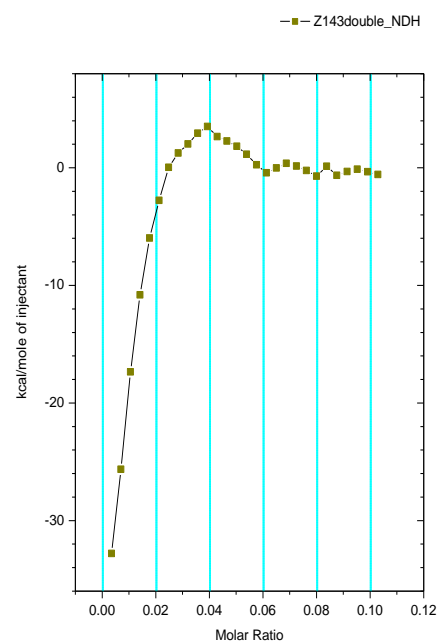
Group A Serum Samples	Anti-PDO IgG Titres	Group B Serum Samples
160, 114	400,000	25, 37, 125
143, 216	250,000-275,000	31, 116
225	150,000	167
213	110,000	120
229, 269	60,000-100,000	129
192	80,000	23, 26
261	35,000	218

**Table 4.11:** The list of serum samples from groups A and B with related IgG titres against PDO used for ITC experiments

1)

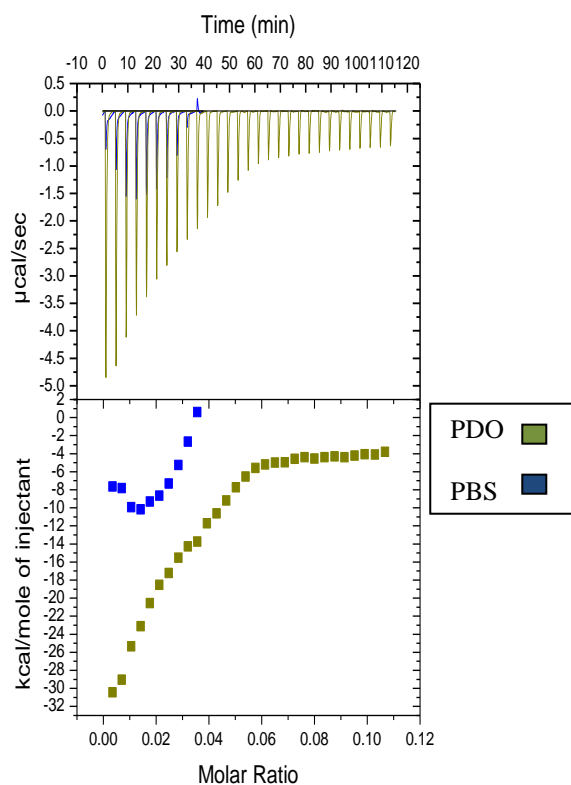


2)

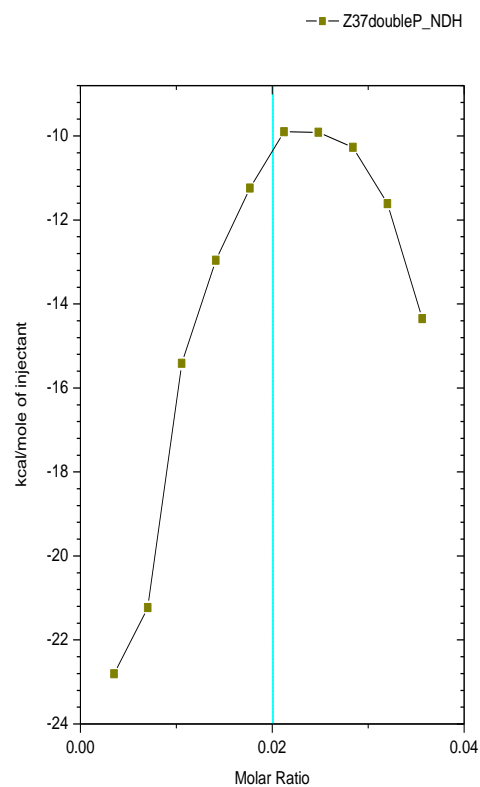


**Figure 4.47:** 1) The comparison results of ITC for sample 143 against PDO at 2 mg/ml and sample 143 against PBS buffer as control test. 2) The final graph for sample 143 after subtraction of control from the main graph.

1)



2)



**Figure 4.48:** 1) The comparison results of ITC for sample 37 against PDO at 2 mg/ml and sample 37 against PBS buffer as control test. 2) The final graph for sample 37 after subtraction of control from the main graph.

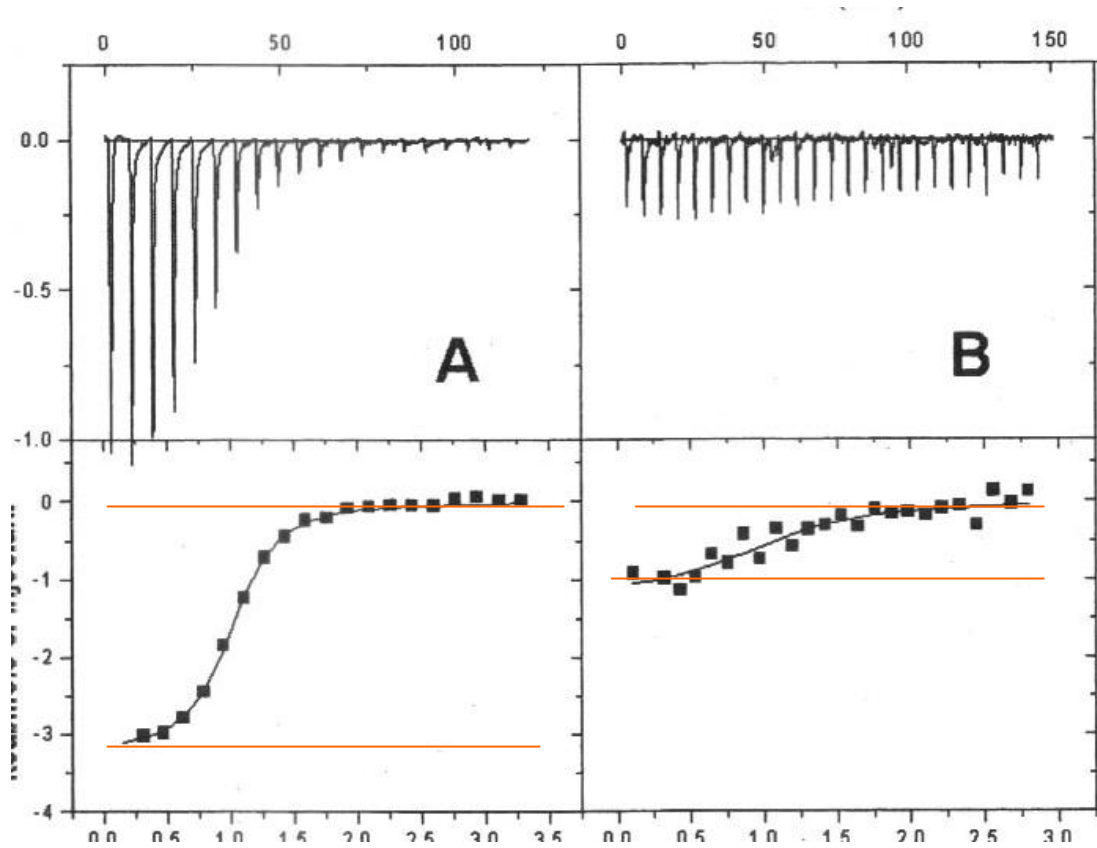
The slope of each graph represents the avidity of the anti-PDO antibody in each serum sample, with a sigmoid slope showing a higher avidity of antibody.

Figure 4.49 is a schematic diagram which gives a better understanding of  $\Delta H$  and how the shape and height of the graph can represent the strength of the reactions and the antibody avidity.  $\Delta H$  is the difference between the 2 plateaux.

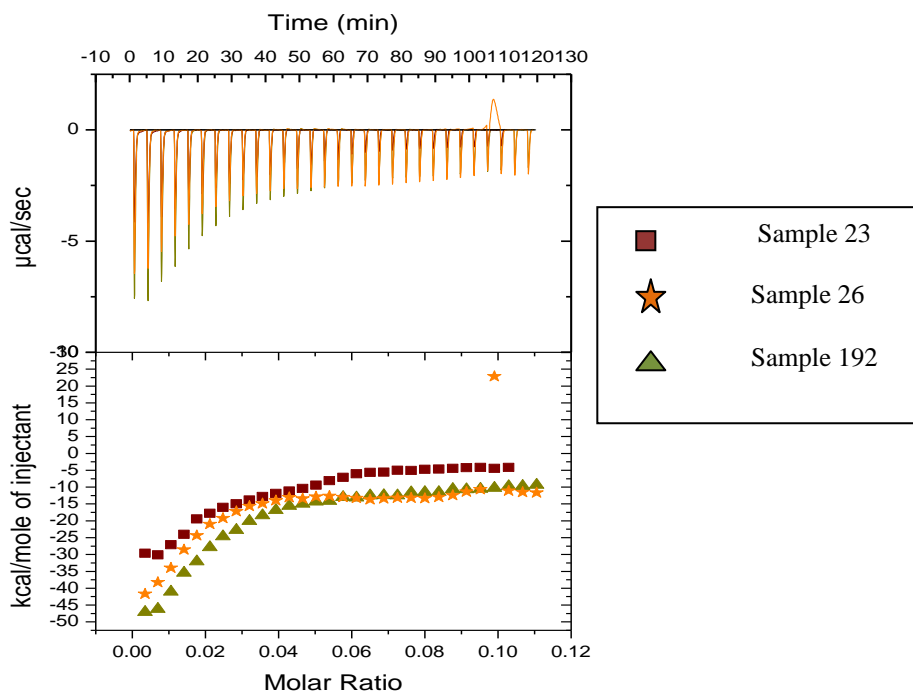
The slope of the graph and the  $\Delta H$  of each serum sample from group A were compared against the related serum sample from group B and the results are shown in figures 4.50 - 4.57 whilst table 4.12 shows the  $\Delta H$  calculated for each sample. 3 arbitrarily cut off points were selected for  $\Delta H$  in order to calculate the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), and also group A and B were compared by Chi- square Test (Table 4.13).

Interestingly, the results showed that the majority of tested serum samples from group A had a higher avidity as measured by  $\Delta H$  than the related serum samples from related samples from group A. In addition, when  $\Delta H \geq 32$  was considered in order to compare the antibody avidity of serum samples from group A with group B, it seems that samples from group A had a significant higher  $\Delta H$  as compared to group B ( $P=0.044$ ) with sensitivity of 80% and specificity of 63%. Both  $\Delta H > 30$  and  $\Delta H \geq 35$  were not significant as samples from group A were compared with samples from group B.

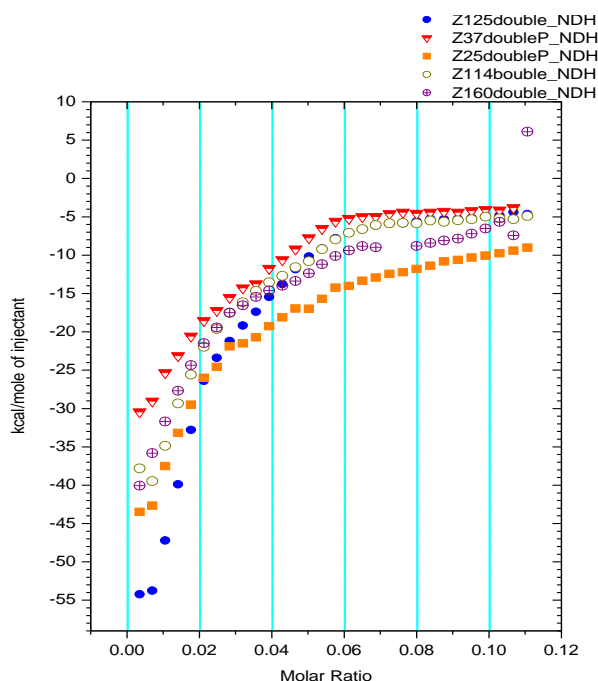




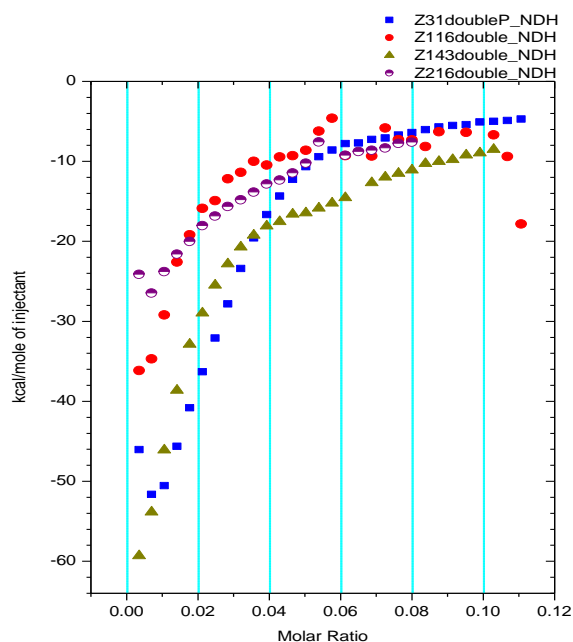
**Figure 4.49:** Schematic graphs showing how to define  $\Delta H$ ;  $\Delta H$  is the difference between the 2 plateaux. Serum A has a higher avidity, binding enthalpy and  $\Delta H$  compared to serum B.



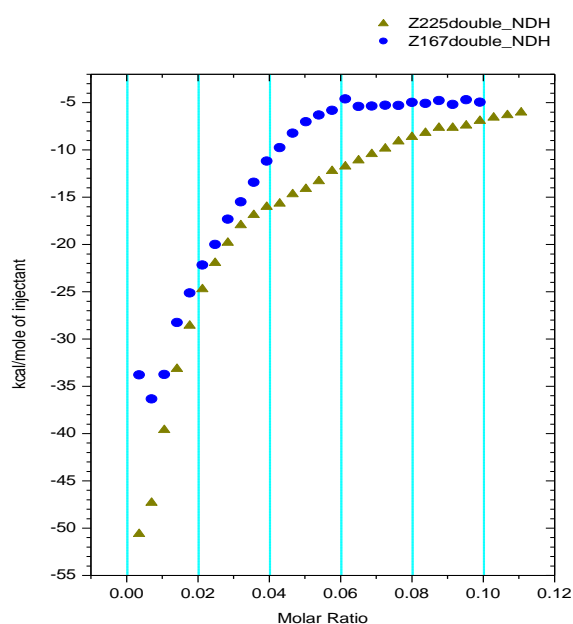
**Figure 4.50:** Comparison between the results of ITC for sample 192 (group A) against PDO at concentration of 2 mg/ml with samples 23 and 26 (group B) against the same pigeon antigen; Sample 192 had a higher  $\Delta H$  compared to samples 23 and 26.



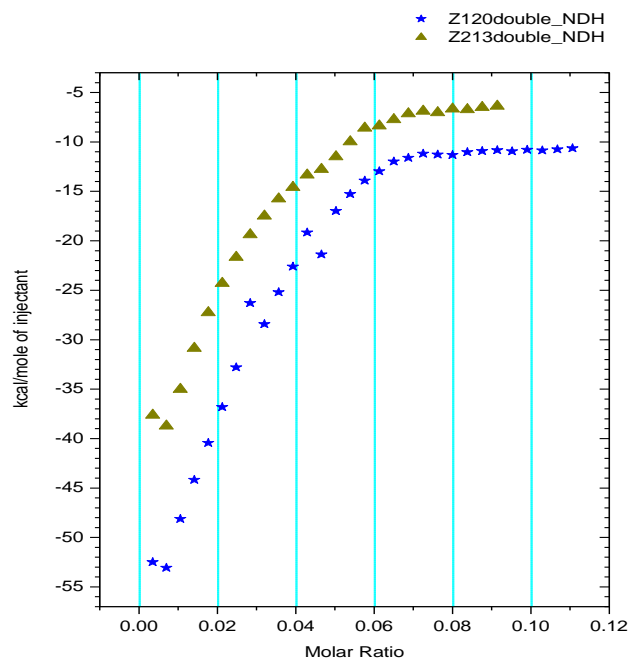
**Figure 4.51:** Comparison between the results of ITC for samples 114 and 160 (group A) against PDO at concentration of 2 mg/ml with samples 25, 37 and 125 (group B) against the same pigeon antigen; Samples 125 and 25 had higher  $\Delta H$  compared to the other samples.



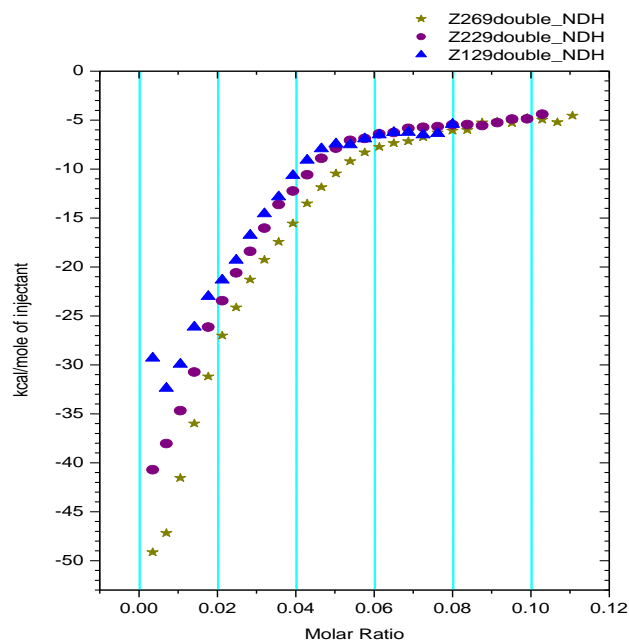
**Figure 4.52:** Comparison between the results of ITC for samples 143 and 216 (group A) against PDO at concentration of 2 mg/ml with samples 31 and 116 (group B) against the same pigeon antigen; Sample 143 had a higher  $\Delta H$  compared to the other samples.



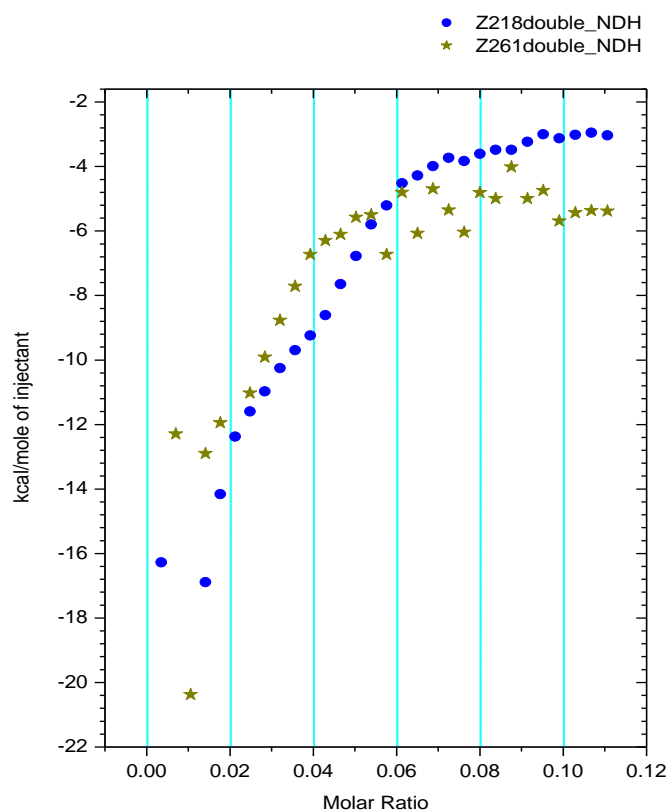
**Figure 4.53:** Comparison between the results of ITC for sample 225 (group A) against PDO at concentration of 2 mg/ml with sample 167 (group B) against the same pigeon antigen; Sample 225 had a higher  $\Delta H$  compared to sample 167.



**Figure 4.54:** Comparison between the results of ITC for sample 213 (group A) against PDO at concentration of 2 mg/ml with sample 120 (group B) against the same pigeon antigen; Sample 120 had a higher  $\Delta H$  compared to sample 213.



**Figure 4.55:** Comparison between the results of ITC for samples 229 and 269 (group A) against PDO at concentration of 2 mg/ml with sample 129 (group B) against the same pigeon antigen; Samples 269 and 229 had higher  $\Delta H$  compared to sample 129.



**Figure 4.56:** Comparison between the results of ITC for sample 261 (group A) against PDO at concentration of 2 mg/ml with sample 218 (group B) against the same pigeon antigen; Samples 261 had higher  $\Delta H$  compared to sample 218.

Samples (Group A)	$\Delta H$	Samples (Group B)	$\Delta H$
*192	35	*26	27
		*23	25
*114	35	*125	49
*160	33	*25	33
		*37	27
*143	51	*31	47
*216	18	*116	28
*225	46	*167	31
*213	32	*120	45
*229	36	*129	27
*269	44		
*261	16	*218	14

**Table 4.12:**  $\Delta H$  for each serum sample

$\Delta H$	sensitivity	specificity	PPV	NPV	Chi-square P value
$>30$	80%	54%	61%	75%	1.04
$\geq 32$	80%	63%	66%	77%	0.044
$\geq 35$	60%	72%	66%	66%	0.13

**Table 4.13:** The results of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for 3 different cut off points of  $\Delta H$ .

#### **4.4: Discussion:**

Bird fanciers' lung is one of the most common forms of HP in the UK (Bourke *et al.*, 2003). There are 80000 registered pigeon fanciers in the UK (McSharry *et al.*, 2006a) and an estimated 5-6 million homes with caged birds such as budgerigars and canaries (Hendrick, Faux & Marshall, 1978; Bourke *et al.*, 2003; Judson & Sahn, 2004). Although the mechanisms involved in the pathogenesis of PFL are unclear, it is generally accepted that the early acute stages of the disease are mediated by immune complexes in the alveolar epithelial surface (Kaltreider, 1993; Razin, Pecht & Rivera, 1995; Bourke *et al.*, 2001). A factor supporting the role of immune complexes in early disease is that the majority of individuals with acute PFL have high levels of systemic and local anti-avian antibodies (Todd, Coan & Allen, 1993; Baldwin *et al.*, 1998b). However, this does not explain why a large number of pigeon fanciers are asymptomatic despite the presence of high levels of anti-avian antibodies in their serum and why a small number of fanciers develop symptoms while having low levels of these antibodies.

It has been suggested that the ability of immune complexes to induce disease will be influenced by all or a number of the following: i) the IgG isotype responses against the causative antigens; ii) the functional affinity of each isotype; and iii) the biochemical nature of the offending antigens (Baldwin *et al.*, 1998c; 1999). A major theme of the study was therefore to investigate antibody responses in PFL. The aim of this section being to investigate anti-avian IgG and IgG subclasses responses among symptomatic and asymptomatic pigeon fanciers and particularly to determine the functional affinity of anti-pigeon antigen IgG isotypes by both inhibition ELISA and isothermal titration calorimetry (ITC). The purpose was to investigate whether there is a significant difference in the functional affinity of specific antibodies between these 2 groups of pigeon fanciers.



PIM is a very large highly glycosylated molecule containing epitopes recognised by the major IgG subclasses and has been previously implicated as a major antigen in the development of PFL (Todd, Coan & Allen, 1991; Todd, Coan & Allen, 1993; Baldwin *et al.*, 1999; Baldwin *et al.*, 2000b). Specific glycosidases of microbial origin are necessary for degradation of oligosaccharide chains of mucin molecules in humans (Corfield *et al.*, 1996) and therefore immune complexes containing mucin may be persistent and problematic in the lung. PIM is present in a various materials inside the pigeon lofts including pigeon droppings and pigeon bloom (Baldwin *et al.*, 1998a; McSharry, Anderson & Boyd, 2000). Therefore, a range of different pigeon antigens containing pigeon mucin i.e. TM, PDO, PDF and PIS together with pigeon mucin itself and PS, which may contain glycoproteins with terminal sugar groups similar to that found in pigeon mucin (Baldwin *et al.*, 1999; Suzuki *et al.*, 2003) have been used in this study. This was the first study looking at IgG subclass responses to TM, PDO, PDF and PIS in PFL.

Firstly the IgG, IgG1 and IgG2 titres against these pigeon antigens have been determined in the five clinical groups (groups A-E). Each group contained 25 pigeon fanciers except group E which included 10 individuals with no previous history of pigeon contact. IgG3 and IgG4 responses were not measured against these antigens as previous studies have shown that IgG3 and IgG4 responses against a range of pigeon antigens are negligible (Todd, Coan & Allen, 1993; Baldwin *et al.*, 1998c).

Previous studies have shown that both symptomatic and asymptomatic antibody positive pigeon fanciers have similarly high IgG titres to both PS and PIM, however, in this study the median titres of IgG against pigeon antigens were always higher in group B than group A and these differences were significant for anti-TM IgG ( $P=0.026$ ) and anti-PDO IgG ( $P=0.036$ ). This suggests that the magnitude of the total IgG response cannot determine the development of the disease although it may be that high levels of antibodies (particularly

some isotypes) are protective as previously reported that measles neutralising antibodies >500 mIU/ml may prevent symptomatic measles infection (Lee *et al.*, 2000).

Also IgG, IgG1 and IgG2 titres against TM, PDO, PDF, PIM and PIS in both groups A and B were much higher than those responses to PS ( $P \leq 0.001$ ). The results for TM and PIM suggest that mucin is a more effective molecule in stimulating B cells and the production of antibodies than PS. Mucin is highly glycosylated with previous studies showing that most anti-mucin IgG1 and IgG2 responses are anti-carbohydrate in nature (Baldwin *et al.*, 1998c; 1999) indicating that the biochemical nature of this antigen may affect the ability of B cells to respond. Pigeon droppings contain a wide variety of materials such as intestinal secretions, mucin and infectious microorganisms ie *Chlamydia*, *Mycoplasma* and *Influenza* which will not be present in pigeon serum (Calvert *et al.*, 1999; Curtis *et al.*, 2002). They may also have a role as adjuvant effect and influence the antibody production in pigeon fanciers (McGee, Elson & McGhee, 1993; Blom & Hilgers, 2004). Baldwin *et al.* (1998) also reported higher antibody titres against PIM as compared to PS (Baldwin *et al.*, 1998c). The lowest median and range of IgG2 titres was seen against PS as compared to the other pigeon antigens, containing pigeon mucin, which may be explained by the fact that mucin has a high polysaccharide content and IgG2 is generally associated with responses to polysaccharide antigens (Hammarstrom & Smith, 1986; Obukhanych & Nussenzweig, 2006) in contrast to IgG1 which is generally generated against T cell-dependent protein antigens (Rubinstein *et al.*, 1998).

It has been suggested that IgG1 may be of greater pathological significance in the development of PFL as immune complexes containing higher levels of this antibody will be more effective in the activation of the classical complement pathway and binding to FC $\gamma$ R (Baldwin *et al.*, 1998c). Surprisingly, in contrast to previous studies, group A did not have significantly higher IgG1 titres to any of the tested antigens (Baldwin *et al.*, 1998c;

Baldwin *et al.*, 2000b). These differences may be explained by the fact that this study used a different study population, smaller group numbers, or the serum samples were collected at a different time of year. For instance, higher specific antibody titres due to intensive contact with pigeon antigens are seen during the pigeon sporting season in late summer and during the period that pigeons shed feathers in the autumn (Dalphin *et al.*, 1993; Tanaka *et al.*, 1995; Bourke *et al.*, 2003). It could also be explained by the fact that IgG1 may not be as important as first thought in the pathogenesis of disease and further studies about the role of mucin and IgG1 responses to mucin in PFL are urgently needed. The median titres of IgG1 and IgG2 against all the pigeon antigens tested was always higher in group B than group A and these differences were significant for anti-PS IgG1 ( $P=0.04$ ), anti-PDF IgG2 ( $P=0.028$ ), anti-PDO IgG2 ( $P=0.04$ ) and anti-PIS IgG2 ( $P=0.03$ ). This is very interesting since it is generally expected to detect higher antibody titres in symptomatic patients with an immune complex mediated disease (Obwaller *et al.*, 1998; Messias-Reason *et al.*, 2002), however this data emphasizes the fact that the magnitude of the IgG subclasses responses cannot determine the development of PFL.

Furthermore, IgG2 titres against all the tested pigeon antigens were higher than IgG1 in both group A and B although these differences were only significant for TM and PDF ( $P=0.03$  and  $P \leq 0.001$ , respectively). This may be due to the non-identical sensitivity of subclass-specific monoclonal antibodies used in the IgG1 and IgG2 assays (Falconer *et al.*, 1993). It can also be explained by the fact that very large highly glycosylated mucin molecules (carbohydrate composition of 70-80%) are present in all the tested antigens except PS and IgG2 is generally associated with responses to polysaccharide antigens containing multiple repeating epitopes (Todd, Coan & Allen, 1993; Obukhanych & Nussenzweig, 2006) and thus mucin may elicit high titres of IgG2. Previously Baldwin *et al.* (2000) reported that the majority of the IgG1 and IgG2 antibodies in pigeon fanciers are

specific for O-linked oligosaccharides of mucin molecules (Baldwin *et al.*, 2000b). In addition, IgG2 may be more protective against polysaccharide antigens as it has been shown that IgG2 was the most active IgG subclass in the binding and opsonic activity against capsular polysaccharide of *Haemophilus influenza* type b and *Streptococcus pneumoniae* and was effective in reducing infections in patients with primary immune deficiency (Mikolajczyk *et al.*, 2004).

The results also showed that pigeon fanciers in groups A and B had a significant higher IgG, IgG1 and IgG2 responses against tested pigeon antigens as compared to groups C and D and control group (group E) ( $P \leq 0.001$ ). Symptomatic fanciers in group C had very low specific antibody titres which emphasises that the level of antibody may not predict the development of disease and other immunological pathways such as T cell responses and type IV hypersensitivity reaction may be involved in the pathogenesis of disease in these fanciers.

The IgG, IgG1 and IgG2 titres against all tested pigeon antigens were also compared with the smoking status of pigeon fanciers by multiple regression, and there was a significant association between antibody titres and smoking status, with those fanciers who smoked having significantly lower antibody titres than non-smokers ( $P < 0.05$ ). This data supports previous studies (McSharry, Banham & Boyd, 1985; Baldwin *et al.*, 1998b) that smoking inhibits antibody production in pigeon fanciers. This may be due to a number of reasons and it has previously been shown that smoking cigarettes may influence antigen presentation and antibody production (Al-Ghamdi & Anil, 2007), inhibit lymphocyte (Kalra *et al.*, 2000) and fibroblast proliferation (Lahmouzi *et al.*, 2000) and lead to a decrease in the secretion of IL-1, IL-6, IL-12, IFN- $\gamma$  and TNF- $\alpha$  (Matsunaga *et al.*, 2001).

Secondly, the avidity index (AI) of IgG1 and IgG2 against 6 pigeon antigens was determined in groups A and B by the use of a chaotrophic ELISA. Basically affinity is

defined as the strength of interaction between a monovalent antigen and a single antibody-binding region, whilst avidity is the strength of the multivalent interaction between antibodies and their antigens which is an important characteristic of protective immune responses (Harris *et al.*, 2007). It has been reported that the antibody avidity influences  $O_2^-$  production and the complement-fixing activity of immune complexes (Alves *et al.*, 2004). Therefore, antibodies with a higher avidity will generally be more efficient in stimulating the respiratory burst of neutrophils and in the activation of complement by the classical and alternative pathway and the generation of large amounts of C3a and C5a which will cause more phagocytes to migrate into the alveoli (Alves *et al.*, 2004). Several studies have previously reported that high avidity antibody against anti-glomerular basement membranes (GBM) may be important in the pathogenesis of anti-GBM disease (Cui & Zhao, 2005) and high avidity antibody has an important role in experimental neuropathy caused by anti-GM1 antibodies (Comin *et al.*, 2006). This, therefore, highlights the important role that antibody avidity may have in the development of immune complex mediated diseases such as PFL. On the other hand, low avidity antibodies may have an important role in the pathogenesis of systemic disease with circulating immune complexes i.e. SLE with a kidney involvement (Nielsen, 1980).

Interestingly in this study, group A generally had higher AI for IgG1 against pigeon antigens as compared to group B, however, the differences were only significant for PDO ( $P=0.006$ ). Group A also had higher AI for IgG2 against most of pigeon antigens tested with significant differences for IgG2 against PDO and PDF ( $P=0.002$  and  $P\leq 0.001$ , respectively). The AI for IgG2 against PIM was higher in group B than group A, however, this difference was not significant. It has been suggested that the differences in IgG subclass profiles and the functional affinity of each isotype may affect the composition and properties of the immune complexes and consequently the development of disease

(Baldwin *et al.*, 2000b). This is supported in this study as symptomatic fanciers in group A had higher IgG antibody avidities and therefore immune complexes in individuals with PFL may have a stronger composition and bonds and consequently they may be harder to remove once deposited in the lung.

As high avidity antibodies stimulate the respiratory burst of neutrophils and the complement system more effectively they may also, therefore, generate more alveolar damage in symptomatic pigeon fanciers (Alves *et al.*, 2004; Cui & Zhao, 2005). This data, therefore, suggests that although the magnitude of the antibody responses cannot predict the development of disease, the level of AI may be a useful marker as asymptomatic pigeon fanciers in group B had higher IgG1 and IgG2 titres and lower antibody avidities as compared to symptomatic fanciers in group A.

Furthermore, the efficiency of those mononuclear phagocytes responsible for removing the immune complexes depends on the functional capacity of the Fc  $\gamma$ R that recognize the Fc portion of IgG and the receptors for complement (Zuniga *et al.*, 2003). Fc $\gamma$ R polymorphisms and amino acid substitutions within extracellular domains of Fc $\gamma$ R are important in the binding avidity of IgG subclasses and may modulate the susceptibility to particular diseases such as SLE (Hepburn *et al.*, 2006). Abnormal clearance of immune complexes by the phagocytic system is an important factor in the pathogenesis of SLE and R131/H131 polymorphism of Fc $\gamma$ RIIa may affect on the uptake of immune complexes. R131 is the low binding allele for human IgG2 and may contribute to the defect in removal of circulating immune complexes (Zuniga *et al.*, 2003). Thus polymorphism in Fc $\gamma$ RIIa may be one of the reasons why fanciers with high levels of specific antibodies (group B) do not have respiratory symptoms following pigeon contact, whilst individuals with lower levels of specific antibodies do develop disease.

It has previously shown that immune complexes containing pigeon mucin and specific anti-mucin antibodies in symptomatic fanciers can activate complement more strongly than immune complexes generated with sera from asymptomatic fanciers (Khan, 1999). Originally it was suggested that this was due to high levels of IgG1 in the immune complex (Baldwin *et al.*, 1998c) but it seems more likely from these results that high avidity antibodies in symptomatic fanciers may be involved in this enhanced ability to activate the respiratory burst of neutrophils and the complement system generating more tissue damages (Neale *et al.*, 1993; Alves *et al.*, 2004).

Finally, the antibody avidity against PDO was determined in 20 pigeon fanciers from group A and B by isothermal calorimetry (ITC). PDO was used as the antigen in these experiments as it was shown that fanciers in group A have significantly higher AI for IgG1 and IgG2 against PDO in comparison with group B ( $P=0.006$  and  $P=0.002$ , respectively). During the experiments, the thermodynamics of the antigen-antibody reaction and the generated heat ( $\Delta H$ ) were measured by the system (Sottriffer *et al.*, 1999; Mosedale *et al.*, 2006).

ITC has previously been used for the investigation of the pathogenesis of a number of different conditions and diseases eg Alzhimers Disease (Hoyer & Hard, 2008), Parkinsons Disease (Bharathi & Rao, 2007), ovarian cancer (Nikolopoulos *et al.*, 2007), Wilsons disease (Wernimont, Yatsunyk & Rosenzweig, 2004), and Lyme disease (Raibaud *et al.*, 2005). It has also been used for identification of the antigenic determinants of an epitope presenting in lipopolysaccharide of gram negative bacteria causing septic shock such as *Enterobacter* (Muller-Loennies *et al.*, 2003). There have been various studies about developing specific monoclonal antibodies against different microorganisms using ITC. For instance, the thermodynamic constants of reaction between the synthesized peptide sequence and staphylococcal enterotoxin B (SEB) causing food intoxication has been

determined by ITC in order to develop a peptide with the highest affinity to SEB (Soykut, Dudak & Boyaci, 2008). Muller-Loennies et al (2003) also studied Gram-negative bacteria causing septic shock in order to identify the antigenic determinants of an epitope which is present in enterobacterial lipopolysaccharide (LPS) and to recognize it by a cross-reactive monoclonal antibody obtained from *Escherichia coli*, *Salmonella enterica*, and *E. Coli* (Muller-Loennies, Lindner & Brade, 2003). Glycoform modification of immunoglobulin and its effect on antibody affinity has also been studied by ITC. Okazaki et al (2004) reported that the depletion of fucose from human IgG1 improves its affinity for Fc $\gamma$ RIIIa and exhibit higher ADCC suggesting that a glyco-engineering strategy can improve a glycoproteins affinity for its receptors (Okazaki *et al.*, 2004). Previously monoclonal antibodies have been used in ITC experiments against one epitope in order to identify the structural details of different microorganisms, for instance the polysaccharides present in Mycobacterial cell wall (Rademacher *et al.*, 2007). However, in this study antibody avidity which is the strength of the multivalent interaction between antibodies and their antigens has been determined.

This is the first time that ITC has been used to determine the antibody avidity using whole sera in a clinical situation such as pigeon fanciers' lung. The results showed that specific antibodies against PDO in pigeon fanciers in group A had higher  $\Delta H$  than group B. 3 arbitrary cut off points ( $>30$ ,  $\geq 32$  and  $\geq 35$ ) were selected for  $\Delta H$  in order to compare pigeon fanciers in groups A and B. Only a  $\Delta H \geq 32$  showed a significant difference ( $P=0.044$ ) between groups A and B. This data showed that the avidity of specific antibody in group A was higher than group B which confirms the results of the inhibition ELISA and emphasises the important role of high avidity antibody in the development of PFL. In addition to the clinical importance of these high avidity antibodies, ITC may be a novel way to evaluate the clinical status of pigeon fanciers by the measurement of  $\Delta H$ . ITC is a



useful, easy and quick technique to predict the development of PFL. Although it does not have a high sensitivity and specificity (80% and 63%, respectively), further studies on larger groups of fanciers may help to further develop this technique as a diagnostic test. Furthermore, the potential use of ITC in other immune complex mediated diseases should be considered.

Finally to summarise:

- i) Antibody titres against pigeon antigens containing PIM, PDO, PDF, PIS and TM were higher than PS and therefore these antigens seem to be more effective at stimulating B cells for the production of antibodies than PS.
- ii) Anti-avian IgG2 was the main IgG subclasses produced in both symptomatic and asymptomatic fanciers.
- iii) The magnitude of antibody response cannot predict the development of PFL.
- iv) Specific antibodies with high avidity against pigeon antigens may explain why some fanciers with low levels of antibodies develop disease.
- v) ITC is a novel and quick test to determine the avidity of specific antibodies in PFL.
- vi) Further development of ITC may provide a novel and quick practical test which may help in the diagnosis of PFL.
- vii) ITC may be a useful diagnostic tool in other immune complex mediated diseases.

## 5: T cell Responses in Pigeon Fanciers' Lung

### 5.1: Introduction:

The pathogenesis of PFL has been suggested to be due to hypersensitivity reactions to pigeon antigens with disease progression being a complex process involving both arms of the immune response ((Kaltreider, 1993; Bourke *et al.*, 2001; McSharry *et al.*, 2002). After phagocytosis and processing of the antigen by antigen presenting cells (APCs) immunogenic epitopes of the antigen bind within the cleft of MHC class II on the surface of the APCs which can then activate CD4<sup>+</sup> T lymphocytes (Desombere *et al.*, 2005). These CD4<sup>+</sup> T cells can then differentiate into various T helper subsets which can either help in antibody production (Jenkins *et al.*, 2001) or cell-mediated immune function.

Both symptomatic and asymptomatic pigeon fanciers generate both B and T cell responses against pigeon antigens as both precipitating IgG antibodies and antigen specific T cells are detected in fanciers blood (Fink, 1992; Ohkawa *et al.*, 2001). These anti-pigeon antigen antibodies, antigen specific lymphocytes and the development of lymphocytic alveolitis may be found in both symptomatic and asymptomatic pigeon fanciers (Fink, Tebo & Barboriak, 1969; Reynolds *et al.*, 1989).

The acute symptoms of PFL correlate with the development of a neutrophilic alveolitis (Fournier *et al.*, 1985; McSharry *et al.*, 2002) which gradually decreases and is replaced by a CD8<sup>+</sup> T cell lymphocytic alveolitis (Reynolds *et al.*, 1989; Murayama *et al.*, 1993; McSharry *et al.*, 2002). The proportion of CD4<sup>+</sup> T cells then increases and alveolar macrophages are activated leading to the secretion of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 and IL-8 which may lead to fibrosis (Calvert *et al.*, 1999; Barrera *et al.*, 2008). It has been suggested that lung fibrosis in PFL is associated with CD4<sup>+</sup> T cells (Murayama *et al.*, 1993) with macrophages being activated directly by the formation of immune complexes or by T cells. Repeated macrophage activation may lead to

granulomata formation which, therefore, may be considered T cell-mediated (Hensley *et al.*, 1969; Pepys, 1973; Kinet, 1999).

Regulatory cytokines such as IL-10 may also be secreted and these may regulate and reduce inflammatory responses in the lung (Groux *et al.*, 1997; Schuyler *et al.*, 1997).

#### **5.1.1: Antigen Presenting Cells:**

T cells recognize processed antigen in the form of peptides or glycolipids bound to either MHC class I or II or CD1 (Rosat *et al.*, 1999; Obukhanych & Nussenzweig, 2006; Barral & Brenner, 2007).

Exogenous protein and glycoprotein antigens are processed and converted into small peptide fragments in endosomes within APCs such as Dendritic cells (DCs) and macrophages (Machy *et al.*, 2002). These fragments then bind to the cleft within the class II MHC molecules and are presented on the cell surface as a complex which can be recognized by CD4<sup>+</sup> T cells (Engelhard, 1994; Obukhanych & Nussenzweig, 2006). Endogenous antigens are processed in the cytosol and then presented by class I MHC molecules (Grey, Sette & Buus, 1989; Machy *et al.*, 2002).

CD1 is a distinct family of antigen-presenting molecules (MHC-class-I like glycoproteins) encoded on chromosome one which present a range of lipid or glycolipid antigens to effector T cells which have roles in antimicrobial immune responses, antitumour immunity and regulation of tolerance (Sonoda *et al.*, 2007; Terabe & Berzofsky, 2007). Five CD1 isoforms are expressed in humans including CD1a, CD1b, CD1c, CD1d and CD1e (Barral & Brenner, 2007). CD1d molecules present mostly lipid and glycolipid antigens to NKT

cells (Gadola *et al.*, 2002; Barral & Brenner, 2007). NKT cells are a distinct population of mature lymphocytes having a  $CD4^+$  or double negative  $CD4^-CD8^-$  phenotype (Wang *et al.*, 2002; Capone *et al.*, 2003; Takagi *et al.*, 2004). Upon activation NKT cells secrete inflammatory cytokines e.g.  $IFN-\gamma$ , IL-4 and GM-CSF which increases the expression of CD1 on the APCs and consequently builds up immunity against micro-organisms such as mycobacterium and suppresses autoimmunity and graft rejection (Sieling *et al.*, 2000; Ueda *et al.*, 2006). NKT cells are also able to enhance  $CD8^+$  T cells proliferation through secreting IL-4 (Ueda *et al.*, 2006).

Among APCs, DCs function most effectively because they express high levels of class II MHC and the co-stimulatory B7 molecule which interacts with CD28 on T cells (Caux, Liu & Banchereau, 1995; Ndejembi *et al.*, 2006). B cells also play an important role as APC (Chung *et al.*, 2003). Resting B cells are not able to activate naïve T cells because they do not express the B7 molecules but following activation, B cells express higher level of class II MHC together with B7 molecules, which make them able to activate naïve T cells and memory cells (Hodgkin & Basten, 1995; Chung *et al.*, 2003).

### **5.1.2: T cell Differentiation:**

After maturation in the foetal liver and bone marrow pre- thymocytes then penetrate the vessels at the cortico-medullary junction and are found in the subcapsular region of the thymic outer cortex (Fu & Chen, 2004). Thymic cortical epithelial cells secrete stromal-derived factor I (CXCL12 or SDF-I) which promotes the forward movement of the cells which is important for the differentiation of pre- thymocytes into early thymocytes which express the pre- T cell receptor (pre-TCR) and are double negative for  $CD4^-CD8^-$  (DN)

(Chen, 2004; Fu & Chen, 2004). In the outer cortex, TCR gene rearrangement influences on the development into double positive  $CD4^+ CD8^+$  (DP). As thymocytes move to the inner cortex, the thymus- expressed cytokine (CCL25 or TECK) and the related receptor (CCR9) play an important role in T cell transition from DN to double positive (DP) and as a consequence thymocytes express CD4 and CD8 which enhance the thymocyte/MHC interaction. The thymocytes at the cortico-medullary junction are a transient population which express CD69 and remain responsive to CCL25. In the third stage of maturation, the DP thymocytes develop into single positive TCR  $\alpha\beta^+ CD4^+$  (T helper) or TCR  $\alpha\beta^+ CD8^+$  (Cytotoxic) medullary thymocytes based on the engagement either with peptide/MHC II or peptide/MHC I complex, respectively. Finally in the thymic medulla the single positive cells develop further during an additional 2 weeks before they are released to the circulation (Anderson & Perlmuter, 1995; Chen, 2004; Fu & Chen, 2004).

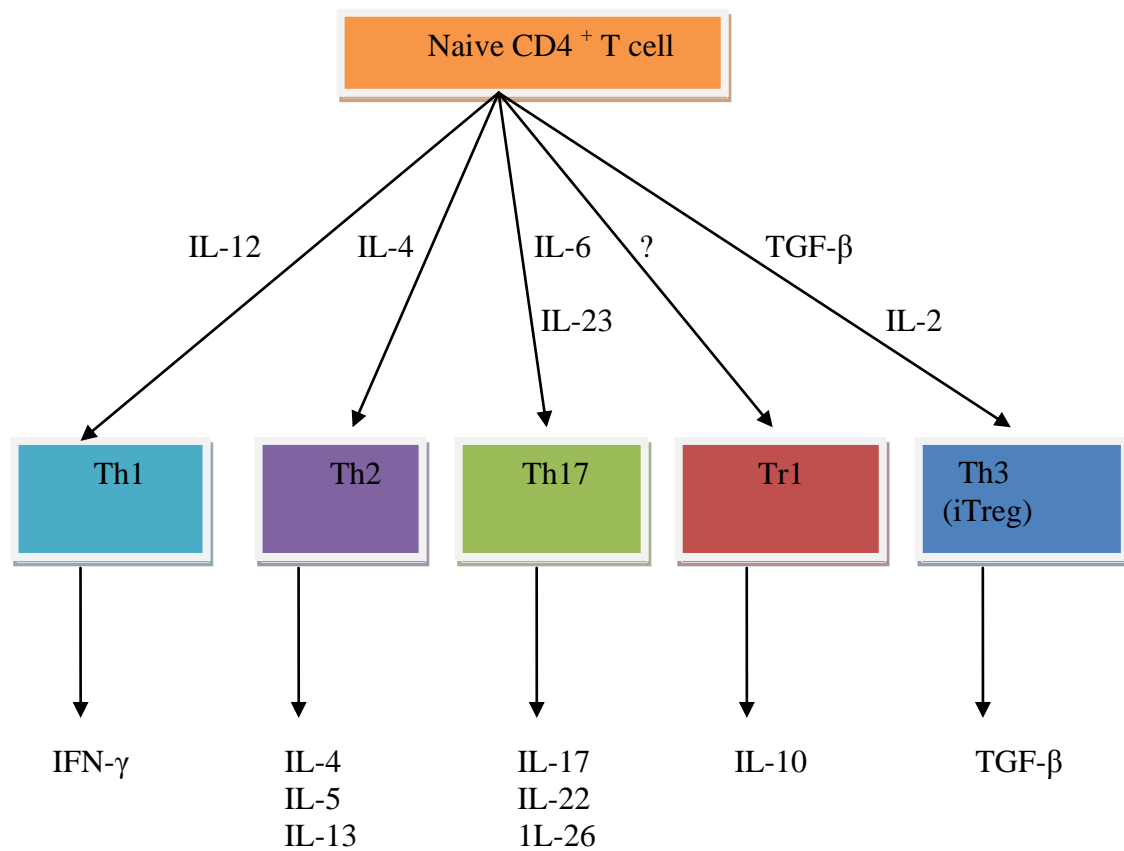
Upon contact with antigen, naive  $CD4^+$  Th cells may differentiate further to Th1, Th2, Th17 and regulatory T cells (Pflanz *et al.*, 2002; Ferretti *et al.*, 2003; Mangan *et al.*, 2006; Oboki *et al.*, 2008). This process can be influenced by antigen concentration, costimulatory molecules and cytokines (figure 5.1) (Pflanz *et al.*, 2002).

Th1 differentiation is initiated by signaling via the TCR and signal transducer and activator of transcription- 1 (STAT-1) - associated cytokine receptors (Anderson *et al.*, 2003). STAT-1 signaling upregulates the transcription factor T-bet which stimulates the inducible chain of the IL-12 receptor (IL-12R $\beta$ 2) on developing Th1 cells (Anderson *et al.*, 2003). As a consequence, IL-12 signaling starts through STAT-4, which leads to IFN- $\gamma$  production by mature Th1 cells (Pflanz *et al.*, 2002). IFN- $\gamma$  can reinforce the whole process through a positive and negative feedback on naive T cells. IFN- $\gamma$  induces STAT1 signaling and T-bet expression in naive  $CD4^+$  T cells leading to upregulation of the IL-12 receptor and suppression of GATA binding protein-3 (GATA-3) (Pflanz *et al.*, 2002). Th1 cells

secreting IFN- $\gamma$  stimulate the immune response to eradicate intracellular pathogens (Anderson *et al.*, 2003).

Th2 differentiation, on the other hand, is initiated by TCR signaling together with IL-4 receptor signaling via STAT-6 which upregulates the expression of the transcription factor GATA-3, a main regulator of Th2 differentiation (Anderson *et al.*, 2003; Sun & Pearce, 2007). In addition, GATA-3 suppresses STAT-4 and the IL-12R $\beta$ 2 chain which are critical to Th1 differentiation, whilst IL-4 produced by mature Th2 cells upregulates GATA-3 via STAT-6 and therefore blocks IL-12R $\beta$ 2 expression. Th2 cells are characterized by secretion of IL-4, IL-5, and IL-13 and they are important in fighting extracellular pathogens (Anderson *et al.*, 2003).

Furthermore, the naive precursor T cells can also be differentiated into Th17 cells in the absence of IFN- $\gamma$  and IL-4, independently of the transcription factors STAT-1, T-bet, STAT-4 and STAT-6 (Ferretti *et al.*, 2003). It has been shown that the differentiation of Th17 cells is induced by the T regulatory cytokine, transforming growth factor-  $\beta$  (TGF- $\beta$ ), together with proinflammatory cytokines e.g. IL-23 and IL-6 (Mangan *et al.*, 2006). TGF- $\beta$  is able to upregulate IL-23R expression and therefore responsiveness to IL-23. IL-17 and IL-23 secreted by Th17 is important in the clearance of several micro-organisms such as *Toxoplasma gondii* (Kelly *et al.*, 2005) and *Pneumocystis carinii* (Rudner *et al.*, 2007). T regulatory cells will be discussed in the following section separately.



**Figure 5.1:** Adopted figure about T cell differentiation in human; naive T cells differentiate into Th1 cells in the presence of IL-12, Th2 in the presence of IL-4, Th17 in the presence of IL-6 and IL-23, Th3 (induced regulatory T cells) in the presence of TGF-β and IL-2. The mechanism of differentiation to type 1 T regulatory cells (Tr1) is still unclear (Oboki *et al.*, 2008).

### 5.1.3: Regulatory T Cells:

Regulatory T cells have a crucial role in the immune system and any defect or impairment in their function can cause autoimmune diseases and atopy (Itoh *et al.*, 1999). They are essential in the down regulation of T cell responses to self and foreign antigens, and the maintenance of T cell homeostasis (Tung *et al.*, 1987; Sakaguchi *et al.*, 1995; Thunberg *et al.*, 2007).

It has been suggested that regulatory T cells (Tregs) have 2 major classes within CD4<sup>+</sup> T cells including:

1. Natural populations or naturally occurring regulatory T cells (nTregs) are generated in the thymus through high affinity recognition of self antigens and express the forkhead-box transcription factor (Foxp3). These are CD4<sup>+</sup> T cells which express surface markers such as IL-2 receptor- $\alpha$  (CD25), the cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the glucocorticoid-induced tumor necrosis factor receptor (GITR) and may constitute 10% of mature CD4<sup>+</sup> T cells in the thymus (Maillard & Snapper, 2007; Maynard *et al.*, 2007; Lehner, 2008). The suppressive function of nTregs initiates with cell-cell contact and depends on IL-10 and TGF- $\beta$  (Sakaguchi, 2000).
2. Induced regulatory T cells (iTregs) or adaptive regulatory T cells (aTregs) are activated in the periphery and their functions are partly cytokine dependent and include:
  - i. Type 1 T regulatory cells (Tr1) which produce regulatory cytokines such as IL-10. Tr1 cells arise in the periphery and their functions are IL-10 dependent (Strauss *et al.*, 2007a; Strauss *et al.*, 2007b).



- ii. Th3 cells which secrete TGF- $\beta$ , have a suppressive function and are able to transfer tolerance (Maillard & Snapper, 2007; Lehner, 2008).

It has been suggested that repetitive stimulation of naïve CD4<sup>+</sup> T cells in the presence of IL-10 helps in the generation of Tr1 cells *in vitro* (Groux *et al.*, 1997; Strauss *et al.*, 2007b). IL-10 is an anti-inflammatory cytokine that inhibits macrophages and DCs resulting in the inhibition of Th1 cell-mediated immunity. It also influence Th2 cells and inhibit cytokine production together with the down-regulation of eosinophil function, reduction of cytokine release by mast cells and suppression of IL-5 production by resting Th0 cells (Moore *et al.*, 2001).

TGF  $\beta$  secreted by Th3 cells, is also an immuno- suppressive cytokine which also helps in IgA production (Chen *et al.*, 1994). Recent investigations on CD4<sup>+</sup> CD57<sup>+</sup> Germinal Centre (GC) T cells have revealed this subset may be a novel type of regulatory cell which suppresses effector cells through cell-cell contact and by the secretion of cytokines including IL-10 and TGF- $\beta$  (Marinova, Han & Zheng, 2007).

#### **5.1.4: B cell Activation:**

B cells may be activated by 2 different routes depending on the biochemical nature of the antigens that stimulate the responses (Jenkins *et al.*, 2001; Lindroth *et al.*, 2004; Obukhanych & Nussenzweig, 2006). These are:

- Thymus dependent (TD) immune responses
- Thymus independent (TI) immune responses

##### **5.1.4.1: Thymus Dependent Antigens:**

TD antigens are protein-based antigens and presented on MHC class II for recognition by Th cells (de Vinuesa *et al.*, 2000; Obukhanych & Nussenzweig, 2006). After uptake of antigen by APC, class II MHC molecules are loaded and the MHC/peptide complexes are transported to the surface of the APC. The additional involvement of co-stimulatory molecules is required to promote the interaction between the DC and T cells (Caux, Liu & Banchereau, 1995; Hodgkin & Basten, 1995; Chung *et al.*, 2003). TCR on Th cells interact with the MHC/peptide complexes resulting in Th cell activation. Activated Th cells will then produce cytokines such as IL-4, IL-5, IL-10 and IL-13 after interaction with B cells (Jenkins *et al.*, 2001). As a result B cells start dividing and differentiate into plasma cells that secrete antibodies. Class switching for the immunoglobulin (Ig) isotypes is an antigen and T cell dependent process which involves class-switch recombination (CSR) and somatic hypermutation (SHM) as described previously in details in section 4.1.1.iii (Imai *et al.*, 2003). In this type of response memory cells derived from B cells have proliferated and undergone affinity maturation in germinal centres (Armitage & Alderson, 1995; Jenkins *et al.*, 2001; Obukhanych & Nussenzweig, 2006).

#### **5.1.4.2: Thymus Independent Antigens:**

TI antigens are divided into 2 types: type 1 and type 2 (Cario *et al.*, 2000; Obukhanych & Nussenzweig, 2006).

Type I – TI (TI-1) antigens such as bacterial LPS are mitogenic stimuli which lead to polyclonal B cell activation via Toll-like receptors- 4 (TLR-4, as a transmembrane co-receptors to CD14) (Cario *et al.*, 2000; Obukhanych & Nussenzweig, 2006). LPS signals via TLRs leading to NF- $\kappa$ B activation (Cario *et al.*, 2000).

Type II – TI (TI-2) antigens are long polymers with repeating units such as bacterial cell wall polysaccharides which have repeating polysaccharide units. These antigens engage B cells by cross-linking surface immunoglobulin receptors (Armitage & Alderson, 1995; Cario *et al.*, 2000; Obukhanych & Nussenzweig, 2006).

In both types of T cell activation there is a little or no class switching from IgM (Lindroth *et al.*, 2004), although IgG2 may be produced, however, memory cells are not generated (Hsu *et al.*, 2006). The mechanisms which control the TI antibody responses are still unclear although there are some suggestions including the necessity of complement receptor engagement in specific targeting of polysaccharide antigens (Guinamard *et al.*, 2000).

#### **5.2: Aims:**

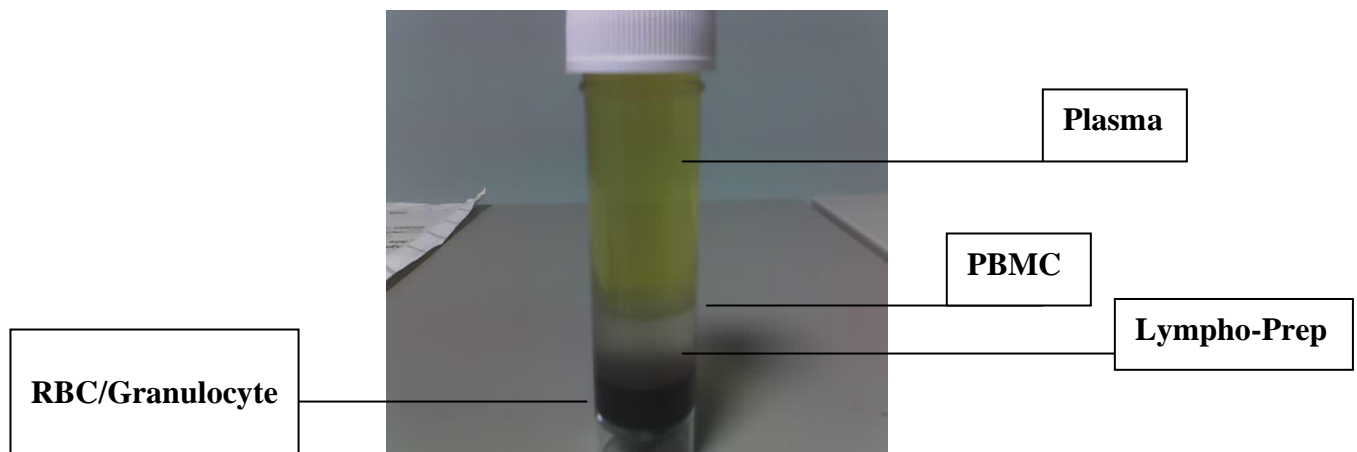
The aim of this section was to generate the mucin-specific T cell clones from pigeon fanciers and to identify the T cell phenotype, cytokine profile and the antigen and epitope specificity of these cells.

### **5.3: Results:**

#### **5.3.1: Generation of Mucin Specific T cell Clones:**

##### **5.3.1.1: Isolation of Peripheral Blood Mononuclear Cells:**

Peripheral blood mononuclear cells were harvested onto Lympho-Prep as previously described in section 3.13 (Figure 5.2).



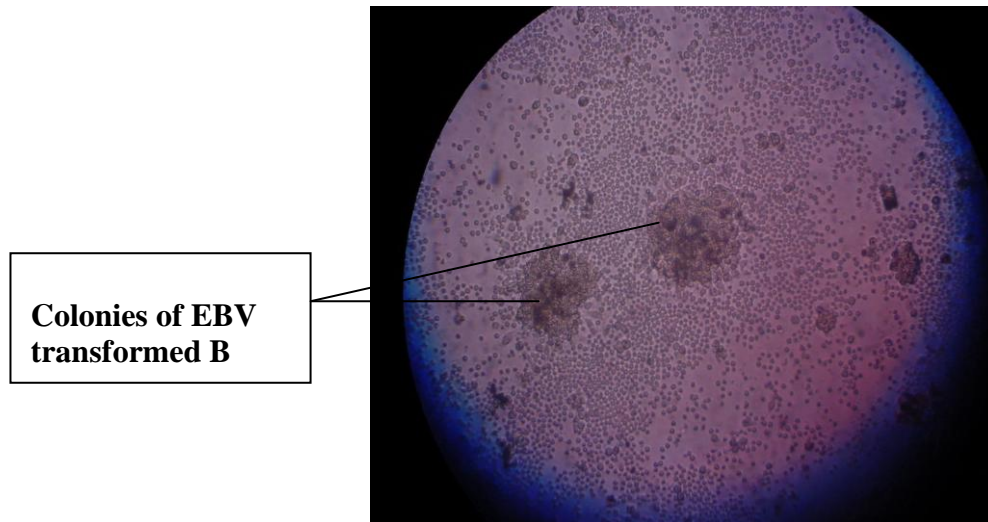
**Figure 5.2:** Universal bottle containing the heparinised peripheral blood and Lympho-Prep after centrifugation.

### **5.3.1.2: Generating of EBV Transformed B cell lines:**

#### **5.3.1.2.i: EBV/ Cyclosporin Transformed B cell lines:**

After incubating PBMC with B958 supernatant containing EBV and cyclosporin

B cells become infected with EBV. Cyclosporin suppresses cytotoxic T cell and natural killer cell activities. Small colonies of transformed cells were seen after a few weeks (Figure 5.3) and after about one month the colonies were transferred to a small culture flask. Finally some of the cells were stored in liquid nitrogen for future use.



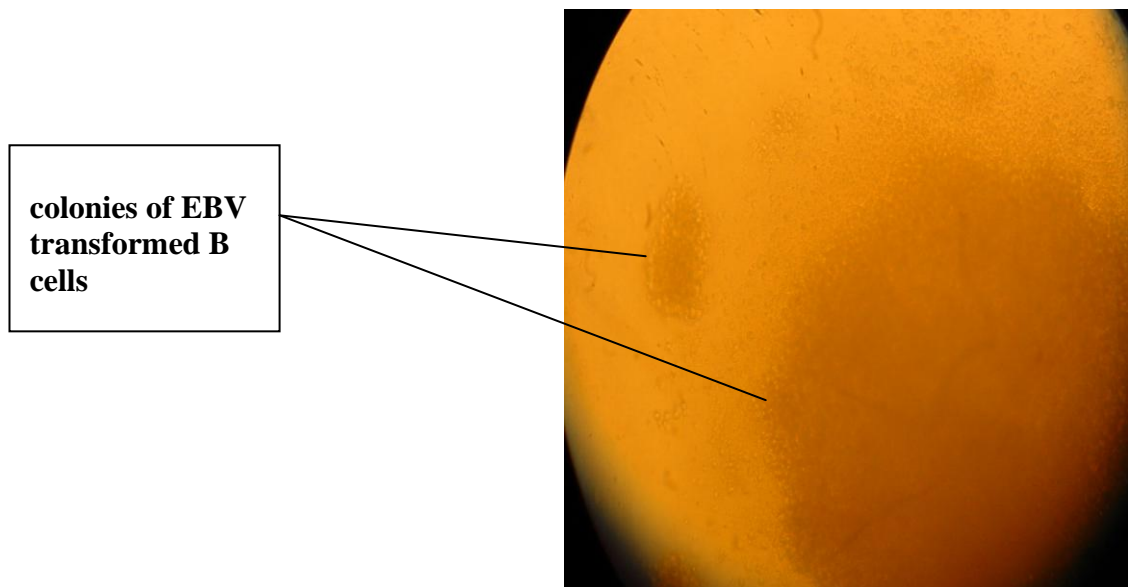
**Figure 5.3:** Colonies of EBV transformed B cells after one week of incubation in RF10 and cyclosporin.

#### **5.3.1.2.ii: EBV/ Polymyxin B Transformed B cells:**

2.5 – 5 x 10<sup>6</sup> freshly separated PBMC were placed in 1.6 ml of transformation medium and the EBV containing supernatant of B958 cell cultures in the ratio of 4:1 (0.4 ml). 1 ml of that mixture was placed into 2 wells and incubated for 4-7 days until visible B cell lines were established (Figure 5.4). Every 3-4 days 1 ml from each well was replaced with a fresh mixture of RF 10. After about 3-4 weeks the cells were transferred to small flask to grow up.

In this method the B cell lines were established in the first week of incubation which was faster, more efficient than cyclosporine method.



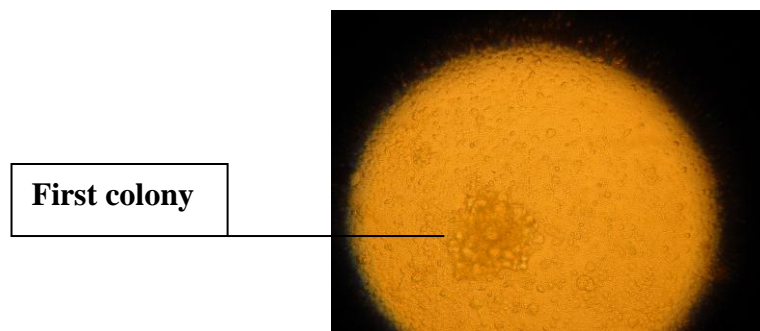


**Figure 5.4:** Colonies of EBV transformed B cells after one week of incubation in transformation medium.

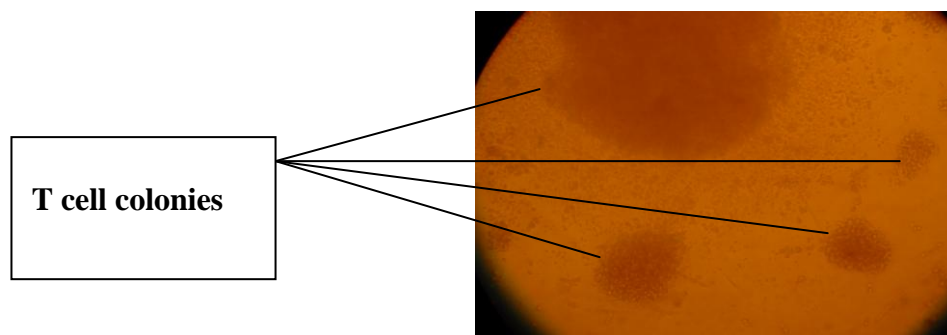
### **5.3.1.3: T cell cloning for Generation of Trypsinised Mucin Specific Clones:**

Dilution cloning is the method of choice for T cell cloning and in this method cells are diluted until there is only one cell in the inoculum's volume. PBMC isolated from an asymptomatic pigeon fancier were stimulated with trypsinised mucin for one week. T cell cultures were then restimulated with trypsinised mucin and the autologous EBV transformed B cell line (feeder cells) was added. Cells were diluted to a concentration of 1 cell/ 20 µl and aliquated into Terasaki plates (GBO, UK). T cell clones were expanded by repeated stimulation with antigen. The first colonies were seen after 2 weeks incubation in Terasaki plates and then they were transferred into 96 well culture plates (Figure 5.5, 5.6 and 5.7). Finally, after 4-6 months there were enough cells to store in liquid nitrogen and for future experiments.

A large number of T cell clones were contaminated with *stereptomyces spp* (Figure 5.8). Finally 21 T cell clones were generated using trypsinised mucin and were later tested for their antigen specificity. One non-specific clone was also generated using PHA to stimulate cells.



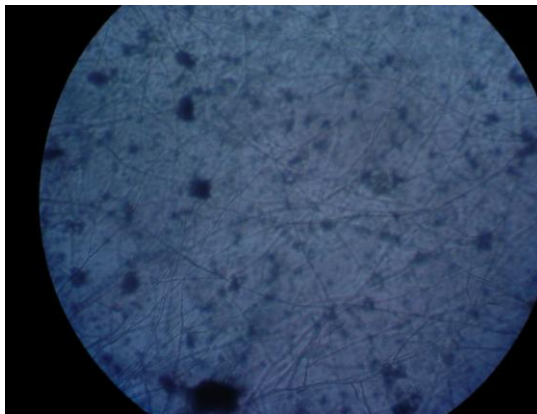
**Figure 5.5:** The colonies after the first 2 weeks incubation



**Figure 5.6:** Colonies after transferring to 96 well culture plate.



**Figure 5.7:** T cell clones in the 96 well culture plate.



**Figure 5.8:** *stereptomycetes* infection of T cell clones.

### 5.3.2: Proliferation Assay:

A dividing lymphocyte requires DNA synthesis thus by the addition of an exogenous source of nucleosides ( $^3\text{H}$ -thymidine) lymphocytes will transport them into the cell. Cell proliferation can then be determined by the incorporation of the  $^3\text{H}$ -thymidine into the dividing cells. Results are defined as stimulation index (SI), whereby the ratio of counts per minute of radioactivity in stimulated cells is divided by the counts per minute for unstimulated cells. In this study a SI of more than 3 was considered to be specific.

Controls for these experiments were IL-2 and PHA which non-specifically stimulate T cells (Lord *et al.*, 2000; Pearson *et al.*, 2001). IL-2 signals through a receptor complex consisting of IL-2R $\alpha$  (CD25), IL-2R $\beta$  (CD122), and  $\gamma$  chain and promotes proliferative signalling which is dependent to tyrosine kinase JAK3 and mediated by STAT-5 (Lord *et al.*, 2000). PHA is a mitogen acting as a polyclonal T cell activator which triggers signal transduction pathway involving mitogen-activated protein kinase which results in mitosis (Pearson *et al.*, 2001).

The SI for the 21 clones generated after stimulation with trypsinised mucin and with the controls is shown in table 5.1.

T cell clones Day 4	IL2 (20ng/ml)	PHA (10µg/ml)	TM (200µg/ml)	TM (50µg/ml)	TM (10µg/ml)
01	37	29	3.3	2.6	3.1
02	43	17	2.9	2.4	3.1
03	51	42	negative	1.25	2.1
04	31	27	6.1	2.3	3.8
05	20	10	2	1.4	2.1
06	15	14	1.8	1.5	2.6
10	50	49	4	3.3	4
13	19	28	2.7	2.4	2.7
14	8	5	1.7	1.2	1.4
15	45	13	1.2	1.1	1.4
16	12	10	1.8	2.1	2.1
17	28	39	5	3.7	4
18	12	8	5.6	3.5	2.6
20	25	11	4.1	2.7	2.1
21	20	21	3.2	2.1	2.6
22	45	43	4.6	3.5	3.8
23	20	27	5.1	3.1	3.2
25	72	11	1.3	1.3	1.7
30	23	36	4.2	3.7	1.8
36	85	39	negative	1.1	1.3
42	28	8	3	3.5	3
Control	32	33	negative	negative	1.1
feeder cells	negative	2	negative	negative	1.3

**Table 5.1:** Stimulation Index (SI) of different T cell clones stimulated with different concentrations of t mucin.  $SI \geq 3$  is shown in bold format.

The SI of T cell clones following stimulation with IL-2 ranged at 8 – 85 with a median of 28. On the other hand, SI after stimulation with PHA ranged at 5 – 49 with a median of 21. Feeder cells did not show any response with IL-2, PHA and t mucin as expected. 8 trypsinised specific T cell clones including 01, 02, 04, 10, 17, 22, 23 and 42 had  $SI \geq 3$  with trypsinised mucin at 10 $\mu$ g/ml whilst 4 t mucin clones including 18, 20, 21 and 30 only proliferated at very high levels of trypsinised mucin. Of these 12 clones only clones 04 and 42 adapted to grow well in RPMI.

### 5.3.3: Cytokine Profile:

T mucin specific and non-specific T cell clones were tested for their ability to produce cytokines in response to the non-specific stimulators (Ionomycin and PMA). Ionomycin is produced by bacterium *Streptomyces conglobatus* and increases the intracellular level of calcium, stimulating the intracellular production of the cytokines (Morgan & Jacob, 1994). PMA is a tumour promoter, activating the signal transduction enzyme protein kinase c (PKC) which stimulates macrophage functions including phagocytosis and the secretion of cytokines (Basta *et al.*, 2001).

$2 \times 10^5$  of each T cell clone was incubated with and without the stimulators (Ionomycin and PMA) in triplicate at 37° C for 1, 2, 3, 4 or 5 days and the supernatant was collected. Results are shown in table 5.2 and t mucin specific clones are underlined.

The detectable limit of each cytokine ELISA was as follows:

IFN $\gamma$  : 68.3 pg/ml, IL-2 and IL-4: 15.6 pg/ml, and IL-5, IL-6 and IL-10: 39 pg/ml.



Cytokines	DAY	<u>Clone 01</u>	<u>Clone 04</u>	Clone 09	Clone 11	Clone 13
<b>IFN<math>\gamma</math> (pg/ml)</b>	1	<68.3	<68.3	<68.3	<68.3	<68.3
	2	<68.3	<68.3	<68.3	<68.3	<68.3
	3	<68.3	<68.3	<68.3	<b>544</b>	<b>600</b>
	4	<68.3	<b>544</b>	<68.3	<b>544</b>	<b>544</b>
	6	<68.3	<68.3	<68.3	<b>2184</b>	<68.3
<b>IL2 (pg/ml)</b>	1	<15.6	<15.6	<15.6	<15.6	<15.6
	2	<15.6	<15.6	<15.6	<15.6	<15.6
	3	<15.6	<15.6	<15.6	<15.6	<15.6
	4	<15.6	<15.6	<15.6	<15.6	<15.6
	6	<15.6	<15.6	<15.6	<15.6	<b>120.8</b>
<b>IL4 (pg/ml)</b>	1	<15.6	<15.6	<15.6	<15.6	<15.6
	2	<15.6	<15.6	<15.6	<15.6	<15.6
	3	<15.6	<15.6	<15.6	<15.6	<15.6
	4	<15.6	<15.6	<15.6	<15.6	<15.6
	6	<15.6	<15.6	<15.6	<15.6	<15.6
<b>IL5 (pg/ml)</b>	1	<39	<39	<39	<39	<39
	2	<39	<39	<39	<39	<39
	3	<39	<39	<39	<39	<39
	4	<39	<39	<39	<39	<39
	6	<39	<39	<39	<39	<39
<b>IL6 (pg/ml)</b>	1	<39	<39	<39	<39	<39
	2	<39	<39	<39	<39	<39
	3	<39	<39	<39	<39	<39
	4	<b>156</b>	<b>156</b>	<39	<39	<39
	6	<39	<39	<39	<39	<b>696</b>
<b>IL10 (pg/ml)</b>	1	<39	<39	<39	<39	<39
	2	<39	<39	<39	<39	<39
	3	<39	<39	<39	<39	<39
	4	<39	<b>94.8</b>	<39	<39	<39
	6	<39	<39	<39	<39	<39

**Table 5.2:** The cytokine levels produced by different clones on different days of incubation period incubating with the stimulators (Ionomycin, PMA). Underlined clones are mucin-specific clones. The < values are below the detectable limit of the ELISA.

Cytokines	DAY	Clone 14	Clone 15	Clone 16	Clone 18	Clone 20
<b>IFN<math>\gamma</math> (pg/ml)</b>	1	<68.3	<68.3	<68.3	<68.3	<68.3
	2	<68.3	<68.3	<68.3	<68.3	<68.3
	3	<68.3	<68.3	<68.3	<68.3	<68.3
	4	<68.3	<68.3	<68.3	<68.3	<68.3
	6	<68.3	<b>3352</b>	<68.3	<68.3	<68.3
<b>IL2 (pg/ml)</b>	1	<15.6	<15.6	<15.6	<15.6	<15.6
	2	<b>46</b>	<15.6	<15.6	<15.6	<15.6
	3	<15.6	<b>62</b>	<15.6	<15.6	<15.6
	4	<15.6	<15.6	<15.6	<15.6	<15.6
	6	<b>167</b>	<b>852</b>	<15.6	<b>135</b>	<15.6
<b>IL4 (pg/ml)</b>	1	<15.6	<15.6	<15.6	<15.6	<15.6
	2	<15.6	<15.6	<15.6	<15.6	<15.6
	3	<15.6	<15.6	<15.6	<15.6	<15.6
	4	<15.6	<15.6	<15.6	<15.6	<15.6
	6	<15.6	<15.6	<15.6	<15.6	<15.6
<b>IL5 (pg/ml)</b>	1	<39	<39	<b>1094</b>	<39	<39
	2	<39	<39	<39	<39	<39
	3	<39	<39	<b>1094</b>	<39	<39
	4	<39	<39	<b>1113</b>	<39	<39
	6	<39	<39	<39	<39	<39
<b>IL6 (pg/ml)</b>	1	<39	<b>78</b>	<39	<39	<39
	2	<39	<b>312</b>	<39	<39	<39
	3	<39	<b>558</b>	<39	<39	<39
	4	<39	<39	<39	<39	<39
	6	<b>732</b>	<b>746</b>	<39	<39	<b>566</b>
<b>IL10 (pg/ml)</b>	1	<39	<b>476.4</b>	<39	<39	<39
	2	<b>81</b>	<39	<39	<39	<39
	3	<39	<39	<39	<39	<39
	4	<b>93.6</b>	<b>94.8</b>	<39	<b>80</b>	<39
	6	<39	<b>533.2</b>	<39	<39	<39

**Table 5.2:** Continued.

Cytokines	DAY	<u>Clone 22</u>	Feeder cells
<b>IFN<math>\gamma</math></b> (pg/ml)	1	<68.3	<68.3
	2	<68.3	<68.3
	3	<68.3	<68.3
	4	<68.3	<68.3
	6	<68.3	<68.3
<b>IL2</b> (pg/ml)	1	<15.6	<15.6
	2	<15.6	<15.6
	3	<15.6	<15.6
	4	<15.6	<15.6
	6	<15.6	<15.6
<b>IL4</b> (pg/ml)	1	<15.6	<15.6
	2	<15.6	<15.6
	3	<15.6	<15.6
	4	<15.6	<15.6
	6	<15.6	<15.6
<b>IL5</b> (pg/ml)	1	<39	<39
	2	<39	<39
	3	<39	<39
	4	<39	<39
	6	<39	<39
<b>IL6</b> (pg/ml)	1	<39	<39
	2	<39	<39
	3	<39	<39
	4	<39	<39
	6	<b>558</b>	<b>586</b>
<b>IL10</b> (pg/ml)	1	<39	<39
	2	<39	<39
	3	<39	<39
	4	<39	<39
	6	<39	<39

**Table 5.2:** Continued.

Most previous studies have determined the cytokine profiles of the cells on single day (Gianfrani *et al.*, 2006; Laughlin *et al.*, 2007; Spaapen *et al.*, 2007; Wolfl *et al.*, 2007), whilst in this study the supernatant of T cell clones were collected after one to six days incubation in order to investigate whether there is a pattern in the production of cytokines by different clones. Clone 04 produced different cytokines in a high level only on day 4 of incubation, however, other T cell clones secreted different cytokines on different days. Among mucin specific T cell clones, clone 02, 10, 17, 21, 23, 30 and 42 also did not have cytokine study due to difficulty in T cell culture and occasional contaminations.

The results showed that IFN- $\gamma$  was produced in a significant level by clone 11 (on day 3, 4 and 6), clone 13 (on day 3 and 4), clone 04 (on day 4) and clone 15 (on day 6). IL-2 was produced significantly by clone 13, 14, 15 and 18 (on day 6), whilst clone 15 also secreted IL-2 on day 3. Clone 16 was the only clone that secreted IL-5 in significant amounts on day 1, 3 and 4. Although IL-6 was produced by the Feeder cells on day 6, the levels of IL-6 produced by clone 13, 14 and 15 (on day 6) were higher. However, clone 15 (on day 1, 2 and 3) and clones 01 and 04 (on day 4) also secreted significant amount of IL-6. In addition, IL-10 was secreted significantly by clone 15 (on day 1, 4 and 6), clone 14 (on day 2 and 4) and clone 04 and 18 (on day 4). A summary of results is shown in table 5.3.

Clones 11 and 13 therefore appear to be Th1 cells, whilst clones 14 and 16 are Th2 cells. On the other hand, clone 15 and 04 were capable of secreting IFN- $\gamma$ , IL-2, IL-6 and IL-10 and therefore look like Th0 cells, whilst clone 18 seems to be a regulatory T cell. Results show that a range of different mucin-specific T cell clones can be generated.

**A**

Day 1	Clone	01	04	09	11	13	14	15	16	18	20	22	feeders
IFN- $\gamma$		-	-	-	-	-	-	-	-	-	-	-	-
IL-2		-	-	-	-	-	-	-	-	-	-	-	-
IL-4		-	-	-	-	-	-	-	-	-	-	-	-
IL-5		-	-	-	-	-	-	-	+	-	-	-	-
IL-6		-	-	-	-	-	-	+	-	-	-	-	-
IL-10		-	-	-	-	-	-	+	-	-	-	-	-

**B**

	Clone	01	04	09	11	13	14	15	16	18	20	22	feeders
IFN- $\gamma$		-	-	-	-	-	-	-	-	-	-	-	-
IL-2		-	-	-	-	-	+	-	-	-	-	-	-
IL-4		-	-	-	-	-	-	-	-	-	-	-	-
IL-5		-	-	-	-	-	-	-	-	-	-	-	-
IL-6		-	-	-	-	-	-	+	-	-	-	-	-
IL-10		-	-	-	-	-	+	-	-	-	-	-	-

**C**

Day 3	Clone	01	04	09	11	13	14	15	16	18	20	22	feeders
IFN- $\gamma$		-	-	-	+	+	-	-	-	-	-	-	-
IL-2		-	-	-	-	-	-	+	-	-	-	-	-
IL-4		-	-	-	-	-	-	-	-	-	-	-	-
IL-5		-	-	-	-	-	-	-	+	-	-	-	-
IL-6		-	-	-	-	-	-	+	-	-	-	-	-
IL-10		-	-	-	-	-	-	-	-	-	-	-	-

**Table 5.3:** Summary of the results of the cytokine profile of T cell clones and feeder cells; A: after one day of incubation, B: after 2 days of incubation, C: after 3 days of incubation, D: after 4 days of incubation, E: after 6 days of incubations.

**D**

Day 4	Clone	01	04	09	11	13	14	15	16	18	20	22	feeders
IFN- $\gamma$		-	+	-	+	+	-	-	-	-	-	-	-
IL-2		-	-	-	-	-	-	-	-	-	-	-	-
IL-4		-	-	-	-	-	-	-	-	-	-	-	-
IL-5		-	-	-	-	-	-	-	+	-	-	-	-
IL-6		+	+	-	-	-	-	-	-	-	-	-	-
IL-10		-	+	-	-	-	+	+	-	+	-	-	-

**E**

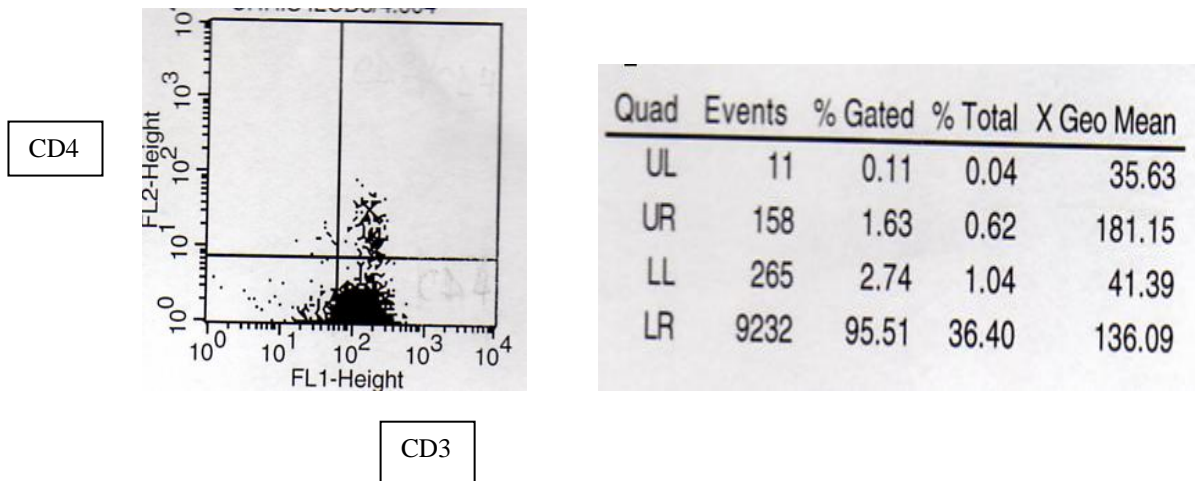
Day 6	Clone	01	04	09	11	13	14	15	16	18	20	22	feeders
IFN- $\gamma$		-	-	-	+	-	-	+	-	-	-	-	-
IL-2		-	-	-	-	+	+	+	-	+	-	-	-
IL-4		-	-	-	-	-	-	-	-	-	-	-	-
IL-5		-	-	-	-	-	-	-	-	-	-	-	-
IL-6		-	-	-	-	+	+	+	-	-	+	+	+
IL-10		-	-	-	-	-	-	+	-	-	-	-	-

**Table 5.3:** Continued.

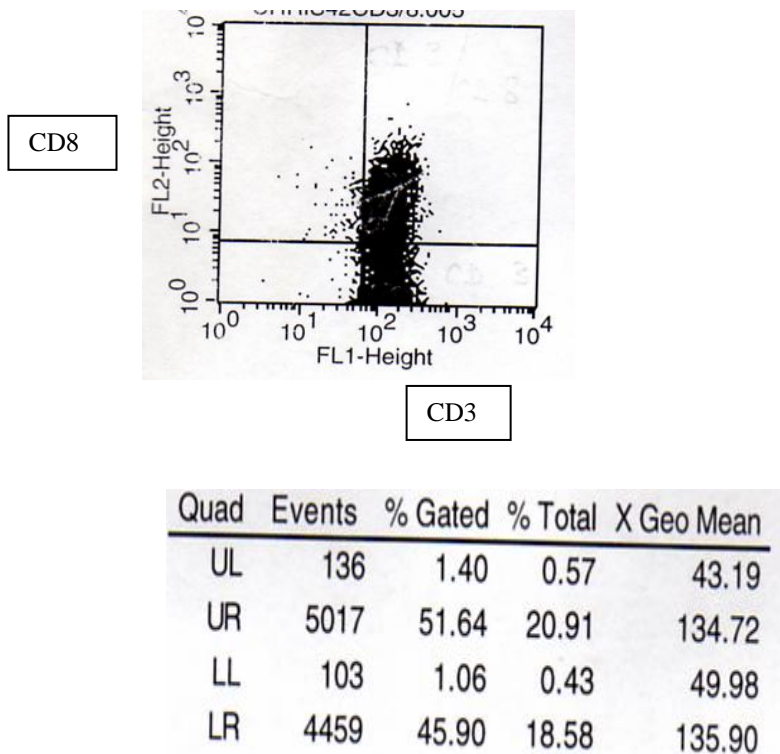
#### **5.3.4: Fluorescence- Activated Cell Sorting Analysis:**

Ten trypsinised mucin specific clones were tested for the cell surface expression of CD3, CD4 and CD8 by FACs analysis using FITC, PE- conjugated monoclonal antibodies. Data of clones 04, 18 and 42 are shown in figures 5.9, 5.10 and 5.11. Table 5.4 shows a summary of the results of FACs analysis.

**Test tube 1:**



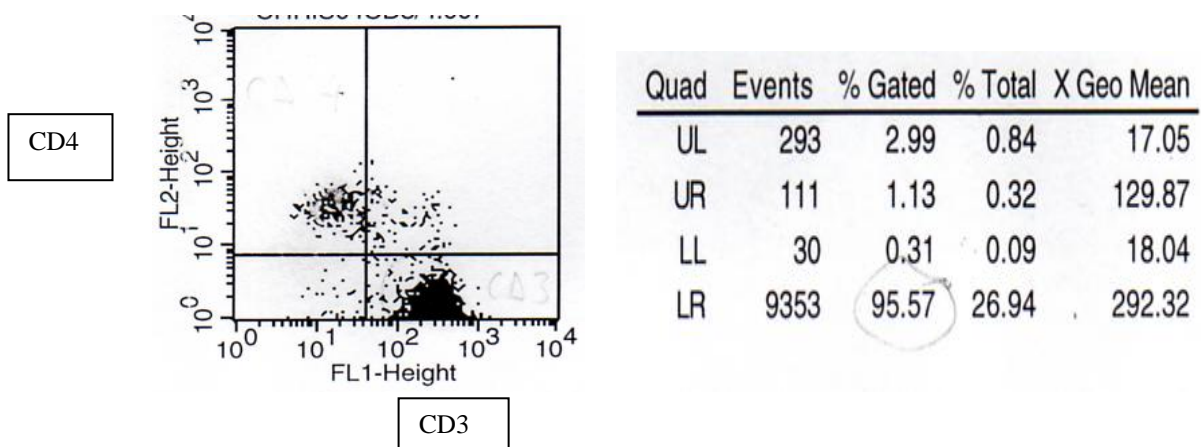
**Test tube 2:**



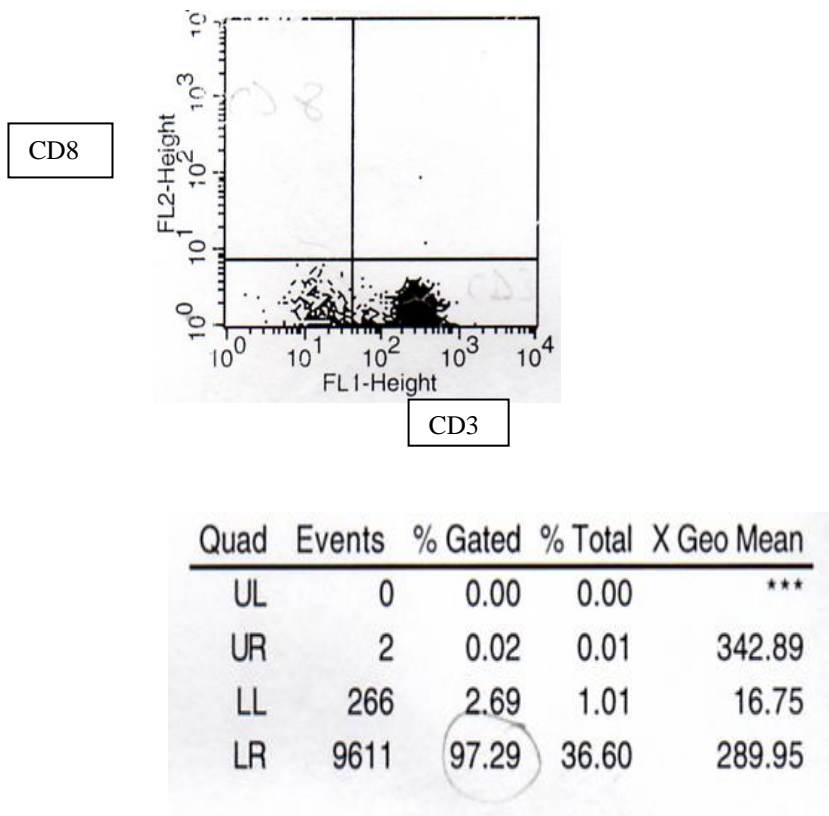
**Figure 5.9:** FACS analysis results for clone 42; T cells are 98% CD3<sup>+</sup> and of these 2% CD3<sup>+</sup> CD4<sup>+</sup>, 96% CD3<sup>+</sup> CD4<sup>-</sup>, 52% CD3<sup>+</sup> CD8<sup>+</sup> and 46% CD3<sup>+</sup> CD8<sup>-</sup>.



**Test tube 1:**

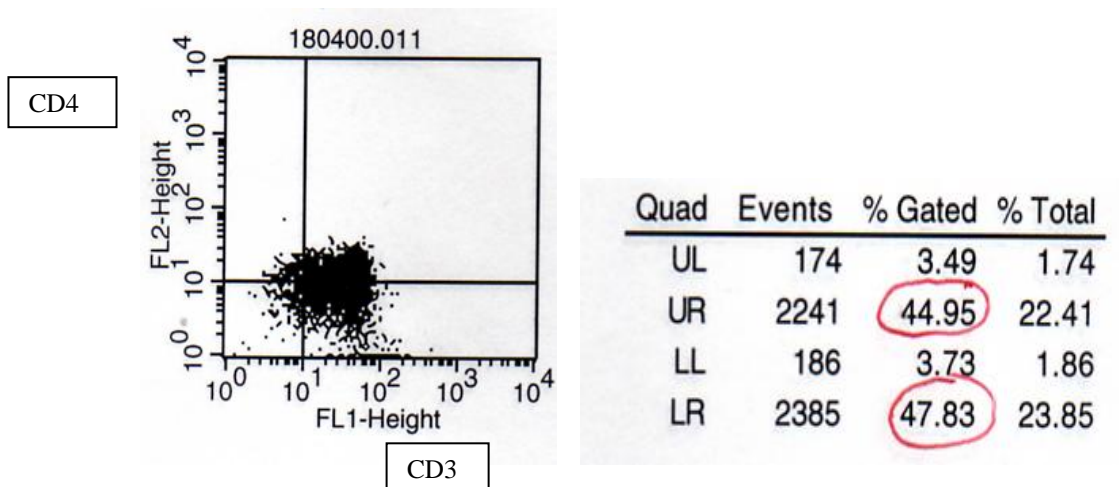


**Test tube 2:**

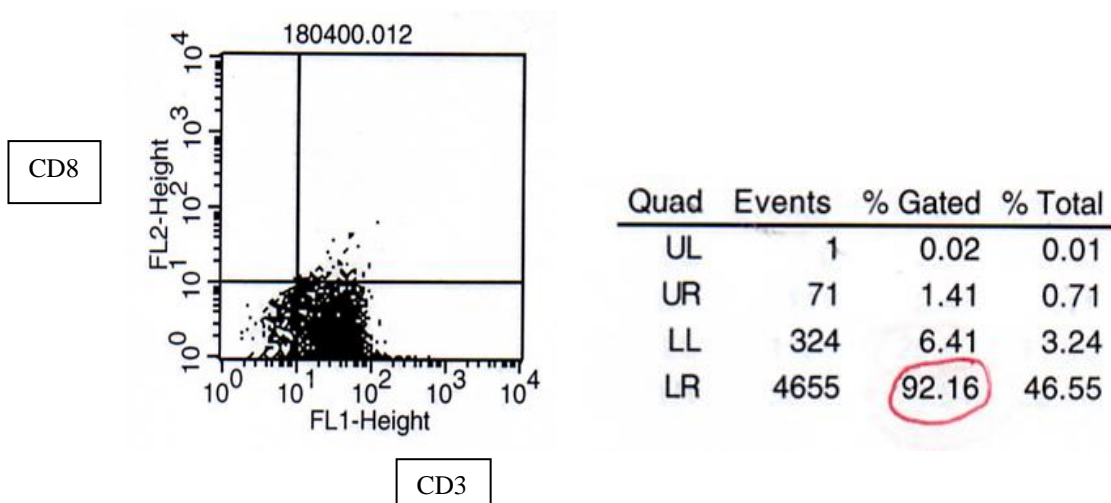


**Figure 5.10:** FACS analysis results for clone 04; T cells are 97% CD3<sup>+</sup> and of these 1% CD3<sup>+</sup> CD4<sup>+</sup>, 96% CD3<sup>+</sup> CD4<sup>-</sup>, 97% CD3<sup>+</sup> CD8<sup>-</sup> and almost no CD3<sup>+</sup> CD8<sup>+</sup>. It appears that clone 04 is a double negative T cell.

**Test tube 1:**



**Test tube 2:**



**Figure 5.11:** FACs analysis results for clone 18; T cells are 93% CD3<sup>+</sup> and of these 45% CD3<sup>+</sup> CD4<sup>+</sup>, 48% CD3<sup>+</sup> CD4<sup>-</sup>, 1% CD3<sup>+</sup> CD8<sup>+</sup> and 92% CD3<sup>+</sup> CD8<sup>-</sup>.

Clone	% CD3 <sup>+</sup>	% CD3 <sup>+</sup> CD4 <sup>+</sup>	% CD3 <sup>+</sup> CD8 <sup>+</sup>	% CD3 <sup>+</sup> CD4 <sup>-</sup> CD8 <sup>-</sup> (double negative)
01	89	18	4	67
04	97	1	0	96
17	98	2	28	70
18	93	45	1	47
20	73	26	2	45
21	93	11	1	81
22	96	4	0	92
23	96	6	0	90
30	97	23	1	74
42	97	2	52	43

**Table 5.4:** Summary of the results of FACs analysis for mucin-specific T cell clones.

Clones 04, 22 and 23 were probably true clones since 90 - 96% of the cells were CD3<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> or double negative (DN). Clones 01, 18, 20, 21 and 30 were probably a mixture of CD3<sup>+</sup> CD4<sup>+</sup> and double negative T cells since a range of 11- 45% were CD3<sup>+</sup> CD4<sup>+</sup> and 45 - 81% were double negative T cells and therefore not true clones. On the other hand, clone 17 and 42 were also a mixture of CD3<sup>+</sup> CD8<sup>+</sup> and double negative T cells since CD3<sup>+</sup> CD8<sup>+</sup> ranged 28 - 52% and double negative T cells ranged 43 – 70%.

This is important as typically cloning technique have a tendency to produce Th1 and Th2 CD4<sup>+</sup> T cells (Jakobsen *et al.*, 2006; Zuercher *et al.*, 2006) and generating CD8<sup>+</sup> or double negative T cells is rare.

#### 5.4: Discussion:

PFL progression is a complex process involving both the humoral and cellular pathways of the immune response (Kaltreider, 1993; Bourke *et al.*, 2001; McSharry *et al.*, 2002) and susceptibility to the causative antigens and host genetic factors are also involved in the pathogenesis of disease (Kurup, Zacharisen & Fink, 2006). The development of clinical symptoms 4-8 hours after antigen exposure and the presence of the precipitating antibodies in pigeon fanciers support the importance of type III hypersensitivity reactions in the development of disease. However, the lack of symptoms in some pigeon fanciers having precipitating antibodies and the absence of vasculitis in the lung biopsy of patients with HP suggest other mechanisms may be involved in the pathogenesis of disease (Kurup, Zacharisen & Fink, 2006). It has been suggested that T cells and inflammatory cytokines may have even greater roles to play in the pathogenesis of disease than immune complexes (Fink, Moore & Barboriak, 1975; Schuyler, Thigpen & Salvaggio, 1978; Curtis *et al.*, 1991; Ohkawa *et al.*, 2001). One of the suggesting evidences is the predominance of neutrophils in BAL fluid in patients with acute HP which is followed by a lymphocytic alveolitis with an increase in CD8<sup>+</sup> T cells and NK cells (Murayama *et al.*, 1993; Kurup, Zacharisen & Fink, 2006) leading to a decrease in the ratio of CD4<sup>+</sup> /CD8<sup>+</sup> T cell to less than one. However, as the disease progresses to the chronic form, an increase in the ratio of CD4<sup>+</sup> /CD8<sup>+</sup> T cell is seen (Irifune *et al.*, 2003; Kurup, Zacharisen & Fink, 2006; Barrera *et al.*, 2008). It has also been suggested that pulmonary macrophages also play an important role in the pathogenesis of disease. They secrete IL-8, a chemotactic factor for neutrophils, and macrophage inflammatory protein (MIP)-1 $\alpha$  which is a chemotactic factor for CD8<sup>+</sup> T cells and promotes further polarisation of CD4<sup>+</sup> T cells to Th1 cells (Denis, 1995; Kurup, Zacharisen & Fink, 2006). Activated macrophages also secrete IL-1 and TNF- $\alpha$  which can stimulate Th1 cells to produce IFN- $\gamma$  which is essential for the

development of disease (Gudmundsson & Hunninghake, 1997; Nance *et al.*, 2005; Matsuno *et al.*, 2007). Further evidence supporting the importance of Th1 cytokines includes the report of transferring experimental HP in mice by Th1 CD4<sup>+</sup> cell lines (Schuyler & Cormier, 1997). Furthermore, NK cells present in BAL and lung tissue of patients with acute HP appear to provide a protective effect (Ratjen *et al.*, 2003).

On the other hand, it has previously been shown that anti-mucin IgG1 was higher in symptomatic fanciers and it was suggested that this response has an important role in the development of disease (Baldwin *et al.*, 2000b). IgG1 is considered to be a T-dependent antibody and thus T cell responses to mucin may be important in the development of disease.

Therefore this study aimed to understand the role of T cells in the pathogenesis of disease and was designed to generate mucin-specific T cell clones from pigeon fanciers and to identify the T cell phenotype, cytokine profile and the antigen and epitope specificity of these cells. This is the first study where T cell clones have been generated to the fully glycosylated mucin molecule. In this study 22 T cell clones were generated from an asymptomatic fancier from group B and 12 of these clones were found to be specific for t mucin. The proliferation assay showed that SI of T cell clones following incubation with IL-2 or PHA was very high (ranged at 5-85) in comparison to the SI of mucin-specific T cell clones after incubation with TM (SI: 3-6). These results suggest although TM is a specific antigen which can stimulate the pigeon fancier's T cell clones to proliferate, it is not as strong as non-specific stimulators such as IL-2 or PHA.

Previous studies reported a mixed Th1/Th2 systemic responses following contact with polysaccharide antigens such as pneumococcal polysaccharide (Jakobsen *et al.*, 2006) and a Th1 response after immunization with O-polysaccharide-toxin A of *P. Aeruginosa* (Zuercher *et al.*, 2006). A regulatory T cell expansion has also been described following

stimulation with ManLAM of *Mycobacterium Tuberculosis* (Garg *et al.*, 2008) whilst a Th0 cytokine response develops after stimulation with *Streptococcus pneumonia* capsular polysaccharide (Mawas, Feavers & Corbel, 2000). Polysaccharide antigens induce endocytosis and delivery into endosomal pathway and it has been shown that mannosylated antigens enhance antigen-uptake and MHC class II antigen presentation by DCs (Tan *et al.*, 1997). Mannan- conjugated tumour antigen mucin1 (MUC1) has also been shown to induce protective Th1/cytotoxic responses; however the Th2/antibody responses showed no protection (Apostolopoulos, Pietersz & McKenzie, 1996; Apostolopoulos *et al.*, 2000). Furthermore, following contact with polysaccharide antigens APCs are activated via Toll-like receptors (TLRs) resulting in activation of NF- $\kappa$ B (transcription factor) which is most often associated with the induction of Th1 responses (Thomas *et al.*, 2005).

Interestingly among the mucin-specific clones generated in this study, clones 04 and 18 produced cytokines which had a Th0 and Treg profile. This shows that regulatory T cells can be generated from peripheral T cells of an asymptomatic pigeon fancier after stimulation with TM in vitro and these cells may play an important role in the prevention of disease in asymptomatic pigeon fanciers. Previously it has also been suggested that regulatory T cells are involved in the regulation of disease (Calvert *et al.*, 1999) and regulatory cytokines such as IL-10 may reduce inflammatory responses in the lung (Groux *et al.*, 1997; Schuyler *et al.*, 1997). Kim *et al.* also reported that glucocorticoid-induced TNF receptor (GITR) on NKT cells may provide costimulatory signals to CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells which modulate immune responses in HP and contributes to the regulation of disease (Kim *et al.*, 2006).

Unfortunately cytokine study was not done for t mucin-specific T cell clones 02, 10, 17, 21, 23, 30 and 42 due to the contamination of the culture plate.

Furthermore, t mucin-specific T cell clones were analyzed by FACs. The results showed clones 04, 22 and 23 were probably true clones since 90 - 96% of the cells were CD4<sup>-</sup> CD8<sup>-</sup> double negative. The rest of the clones were a mixture of CD3<sup>+</sup> CD4<sup>+</sup> or CD3<sup>+</sup> CD8<sup>+</sup> and DN T cells. Recent studies about DN T cells indicates that they are the second most important sources of IFN- $\gamma$  in patients with infection such as cutaneous leishmaniasis and also have an immunoregulatory role to modulate the inflammation (Chen *et al.*, 2004; Antonelli *et al.*, 2006). 75% of DN T cells in healthy individuals may express TCR- $\gamma\delta$  and the balance between the  $\alpha\beta$ - DN T cells expressing proinflammatory cytokines with the  $\gamma\delta$ - DN T cells producing regulatory cytokines is important in formation of an effective cellular response (Antonelli *et al.*, 2006). DN T cells may also have an antitumor role and are effective in prevention of GvHD by inhibiting donor T-cell activation (Young *et al.*, 2003). The FACs results suggest that the presence of DN T cells may be one of the mechanisms that T cells directly conduct to regulate the disease. These cells also may be a source of IFN- $\gamma$  as the cytokine profile of clone 04 showed high IFN- $\gamma$  production in day 4 of incubation.

Unfortunately in this study, the type of TCR on mucin-specific T cell clones was not studied due to contamination of the cultures. However, this data does indicate that t mucin-specific T cell clones with double negative phenotype may have a crucial role in immune regulation in antibody positive asymptomatic fanciers (group B) and can be one of the reasons of why these individuals do not have any symptoms in spite of having high antibody responses.



## 6: Discussion:

PFL is a complex disease involving both arms of the immune response (Kaltreider, 1993; Bourke *et al.*, 2001; McSharry *et al.*, 2002). Improvements in our understanding of the immunopathogenesis of disease depend on advances in our knowledge of the role of different cell types, cytokine profile and affinity of specific antibodies that contribute to protective immunity. In spite of the large number of individuals who have contact with the causative antigens, the prevalence and incidence of HP seems to be less than 10% in the population at risk (Bourke *et al.*, 2003; Fink *et al.*, 2005). Therefore, there may be a number of different defense mechanisms in asymptomatic pigeon fanciers such as i) low avidity antibodies against pigeon antigens and ii) generation of double negative T cells which can prevent the development of symptoms. Therefore, this study was designed to investigate the importance of antibody and T cell responses in pigeon fanciers' lung.

6 pigeon antigens; PIM, TM, PS, PDO, PDF and PIS have been used to study the antibody responses in pigeon fanciers. This is the first time that TM, PDO, PDF and PIS have been studied in this way.

In contrast to the majority of previous studies which investigated antibody responses to pigeon serum (Fink, Tebo & Barboriak, 1969; Rodriguez de Castro *et al.*, 1993; Rodrigo *et al.*, 2000; Aguilar Leon, Novelo Retana & Martinez-Cordero, 2003; McSharry *et al.*, 2006a; McSharry *et al.*, 2006b), this study suggested that TM, PDO, PDF and PIS, which all contain the highly glycosylated mucin molecule, were more effective stimulators of B cells in producing antibodies as compared to PS ( $P \leq 0.001$ ).

Previous studies have shown that anti-mucin IgG1 titres were significantly higher in symptomatic fanciers (Baldwin *et al.*, 1998c; Baldwin *et al.*, 2000b) as compared to asymptomatic fanciers. These results were not replicated in this study and this may be due to the fact that IgG1 may not be important in pathogenesis of the disease. The other factors

which can also be responsible are the size and method of classification of study populations, and the collection of serum samples at different times of the year. Further studies as to the role of mucin and IgG1 responses to mucin are urgently needed to clarify whether antibody responses to this antigen is important in the development of disease. Surprisingly, in this study group B had higher antibody titres against pigeon antigens than group A. This could be due to the different classification of the study group, for instance classifying all symptomatic fanciers regardless of their antibody levels in one group. Also this result together with the fact that some symptomatic fanciers have low levels of these specific antibodies may suggest that the magnitude of the antibody responses cannot determine the development of the disease.

IgG2 titres against all tested pigeon antigens were higher than IgG1 in this study. As IgG2 is generally a TI antibody and associated with responses to carbohydrate antigens (Hammarstrom & Smith, 1986; Obukhanych & Nussenzweig, 2006), it is tempting to suggest that these antibodies may be specific for carbohydrate parts of mucin or other highly glycosylated molecules.

The biological activity and the strength of immune complexes depend on the IgG isotype response, the functional affinity of each isotype and the biochemical nature of related antigens (Baldwin *et al.*, 1998c; 1999). In this study group A had higher avidity index for IgG2 against most pigeon antigens with significant differences for IgG2 against PDO and PDF ( $P=0.002$  and  $P\leq 0.001$ , respectively). These results suggest the presence of high levels of IgG2 with high avidity against pigeon antigens containing mucin may influence the development of PFL in group A since they may have immune complexes with stronger composition which can be harder to remove. In addition antibodies with higher avidity will generally be more efficient in the activation of complement by the classical and alternative

pathway, generation of large amount of C3a and C5a and stimulating the respiratory burst of neutrophils resulting in large amount of damage to lung tissue (Alves *et al.*, 2004).

Other factors such as the functional capacity of Fc $\gamma$ R may also be important in the clearance of immune complexes and amino acid substitutions within the extracellular domains of Fc $\gamma$ R may influence the binding avidity of IgG subclasses (Zuniga *et al.*, 2003). This may be an important area of research in the future.

The avidity of anti-PDO antibodies in some of the pigeon fanciers in groups A and B were determined by ITC. This is the first time that ITC has been used to determine antibody avidity in a clinical situation using whole serum as a source of antibodies. Group A had significantly higher  $\Delta H$  than group B ( $P=0.044$ ) when  $\Delta H \geq 32$  was used as a cut off point. This confirms the results of the avidity index of mucin-specific antibodies determined by inhibition ELISA and also emphasizes the importance of antibody avidity in the pathogenesis of PFL. It also indicates that the measurement of  $\Delta H$  by ITC may be a practical, quick and novel test as part of routine investigations of PFL in clinics. Although it does not have a high sensitivity and specificity (80% and 63%, respectively), further studies on a larger group of fanciers may help to further develop this test. Furthermore, the potential use of ITC in other clinical situations such as immune complex mediated diseases should be emphasised.

Furthermore, T cells and inflammatory cytokines may have even greater roles to play in the pathogenesis of disease than immune complexes (Fink, Moore & Barboriak, 1975; Schuyler, Thigpen & Salvaggio, 1978; Curtis *et al.*, 1991; Ohkawa *et al.*, 2001).

In this study 22 T cell clones were generated from an asymptomatic fancier from group B in which 12 clones were specific for TM. The cytokine profile of these clones showed a range of different T cell subsets can be generated following stimulation with polysaccharide antigen (TM). Interestingly, clone 04 and 18 were a Th0 and a Treg cell.

This is the first time that the cytokine profile of mucin-specific T cell clones has been studied and it shows that regulatory T cells can be generated from peripheral T cells of an asymptomatic pigeon fancier after stimulation with TM and these cells may play an important role in the prevention of disease in asymptomatic pigeon fanciers.

The phenotypes of t mucin-specific T cell clones were analyzed by FACs and these clones were shown to be DN T cells. This generation of DN T cells to t mucin is interesting since DN T cells have an immunoregulatory role and modulate inflammatory immune responses and they are the second most important sources of IFN- $\gamma$  (Antonelli *et al.*, 2006). The cytokine profile of clone 04 showed a high level of IFN- $\gamma$  production on day 4. This data indicates that t mucin-specific T cell clones with a double negative phenotype may also have a crucial role in direct immune regulation in antibody positive asymptomatic fanciers (group B) and may explain why these individuals do not have symptoms in spite of having high antibody responses.

In conclusion, the immunopathogenesis of PFL is complicated and involves both humoral and cell defense mechanisms. The data showed the presence of low avidity specific antibodies together with double negative mucin-specific T cells may be crucial in the prevention of disease development in asymptomatic pigeon fanciers in group B.

## **7: Future Work:**

Further studies may be conducted in order to investigate the role of following aspects in the development of PFL:

1. To study the affinity of specific antibodies in PFL by ITC in more details and bigger study populations in order to optimize the method and to establish an easy and quick diagnostic test with high sensitivity and specificity.
2. To study Fc $\gamma$ R polymorphisms and their roles in binding avidity of IgG subclasses in PFL.
3. To study TCR properties of mucin-specific T cell clones and their role in the pathogenesis of PFL.
4. To study the role of antigen presenting cells in presentation of mucin to T cells.
5. To study the role of regulatory T cells in the pathogenesis of PFL.

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## Appendix I: List of Suppliers

- **Sigma Aldrich Company Ltd:** Gillingham, UK
- **Pharmingen, BD Bioscience:** Oxford, UK
- **Maxisorp, Nunc:** Roskilde, Denmark
- **Dako cytometry:** Glostrup, Denmark
- **Nycomed Pharma:** Asker, Norway
- **Invitrogen, GIBCO:** Paisley, UK
- **MicroCal LLC:** Milton Keynes, UK
- **BDH:** Bath, UK
- **Bio-Rad:** Hemstead, UK
- **MicroCal LLC:** Milton Keynes, UK
- **MDS Nordion:** Ottawa, Canada
- **Packard Instrument Company:** Meriden, CT, USA
- **NIBSC:** Hertfordshire, UK
- **Bio-TEK Instruments:** Vermont, USA
- **Fisher Scientific:** Leicestershire, UK
- **Sandoz:** Holzkirchen, Germany
- **Greiner Bio-One Co. Ltd (GBO):** Business Park, Stonehouse, UK
- **Becton Dickinson UK Ltd:** Abingdon, UK

## Appendix II: Buffers

- **Phosphate Buffer Saline PH =7.0 (PBS)**

NaCL: 8.17 g, Na<sub>2</sub>HPO<sub>4</sub>: 1.21 g, NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O: 0.22 g, Distilled H<sub>2</sub>O (dH<sub>2</sub>O): 1 Litre.

- **PBS-Tween (PBS-T) for washing for ELISA**

PBS / 0.05% Tween 20 (PBS: 1 Litre, Tween: 0.5 ml).

- **Coating Buffer for ELISA**

0.1 M Sodium Phosphate, PH=7.0 (Na<sub>2</sub>HPO<sub>4</sub>: 8.7 g, NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O: 5.37 g, d H<sub>2</sub>O: 1 Litre).

- **PBS-Tween- BSA (PBS-T-BSA) for blocking for ELISA**

PBS / 1% Bovine Serum Albumin (BSA) / 0.05% Tween 20 (PBS: 1 Litre, BSA: 10 g, Tween: 0.5 ml).

- **Citrate Phosphate Buffer for ELISA**

Citric Acid: 5.1 g, Na<sub>2</sub>HPO<sub>4</sub>: 7.31 g, d H<sub>2</sub>O: 1 Litre.

- **Substrate Solution for ELISA**

Citrate Phosphate: 25 ml, Orthophenylenediamine (OPD): 400 µl, H<sub>2</sub>O<sub>2</sub>: 25 µl

- **EDTA PH=8.0**

Na<sub>2</sub>EDTA: 186.1 g, d H<sub>2</sub>O: 500 ml.

Dissolve in approximately 400 ml d H<sub>2</sub>O, adjust PH to 8.0 with 10 N NaOH and make up to 1 litre final volume with d H<sub>2</sub>O.

### **Appendix III: Serial Dilution of Ammonium Thiocyanate**

- 10 M Ammonium Thiocyanate: 10 ml PBS + 7.61 g Ammonium Thiocyanate
- 9 M Ammonium Thiocyanate: 10 ml PBS + 6.85 g Ammonium Thiocyanate
- 8 M Ammonium Thiocyanate: 10 ml PBS + 6 g Ammonium Thiocyanate
- 7 M Ammonium Thiocyanate: 10 ml PBS + 5.32 g Ammonium Thiocyanate
- 6 M Ammonium Thiocyanate: 10 ml PBS + 4.56 g Ammonium Thiocyanate
- 5 M Ammonium Thiocyanate: 10 ml PBS + 3.8 g Ammonium Thiocyanate
- 4 M Ammonium Thiocyanate: 10 ml PBS + 3 g Ammonium Thiocyanate
- 3 M Ammonium Thiocyanate: 10 ml PBS + 2.28 g Ammonium Thiocyanate
- 2 M Ammonium Thiocyanate: 10 ml PBS + 1.52 g Ammonium Thiocyanate
- 1 M Ammonium Thiocyanate: 10 ml PBS + 0.76 g Ammonium Thiocyanate

#### **Appendix IV: Dilutions of Anti-Cytokine Antibodies for ELISA**

Anti-cytokine antibodies (Pharminogen, Oxford,UK) were diluted in coating buffer appropriately as below:

IL-2	IL-4	IL-5	IL-6	IL-10	IFN- $\gamma$	TNF- $\alpha$	GM-CSF	TGF- $\beta$
1/200	1/500	1/1000	1/4000	1/1000	1/500	1/1000	1/500	1/800



## **Appendix V: Final Concentration of Top Standards for ELISA**

Top standards (NIBSC, Hertfordshire, UK) were diluted in PBS-T-BSA and final concentration of each top standard is as following:

- IL-2: 2000 pg/ml (stock concentration: 10 ng/ml)
- IL-4: 2000 pg/ml (stock concentration: 20 ng/ml)
- IL-5: 5000 pg/ml (stock concentration: 50 ng/ml)
- IL-6: 5000 pg/ml (stock concentration: 200 ng/ml)
- IL-10: 5000 pg/ml (stock concentration: 100 ng/ml)
- IFN- $\gamma$ : 8750 pg/ml (stock concentration: 175 ng/ml)
- TNF- $\alpha$ : 10000 pg/ml (stock concentration: 100 ng/ml)
- GM-CSF: 10000 pg/ml (stock concentration: 10000 ng/ml)
- TGF- $\beta$ : 250000 pg/ml (stock concentration: 5000 ng/ml)

## **Appendix VI: Dilutions of Biotinylated Anti-Cytokine Antibodies for ELISA**

Biotinylated anti-cytokine antibodies (Pharmingen, Oxford ,UK) were diluted in PBS-T-BSA appropriately as below:

IL-2	IL-4	IL-5	IL-6	IL-10	IFN- $\gamma$	TNF- $\alpha$	GM-CSF	TGF- $\beta$
1/400	1/1000	1/1000	1/4000	1/1000	1/2000	1/1000	1/200	1/400

## Glossary:

- $\Delta H$ : Heat generated by the antigen-antibody interactions
- $^3\text{H}$ -thymidine: Tritiated thymidine
- ADCC: Antibody-dependent cell mediated cytotoxicity
- AI: Avidity index
- AID: Activation-induced cytidine deaminase
- APCs: Antigen presenting cells
- AT: Ammonium thiocyanate
- aTregs: Adaptive regulatory T cells
- B: Factor B
- BAL: Bronchoalveolar lavage
- BCG: Bacille Calmette-Guerin
- BDP: Beclomethasone dipropionate
- BSA: Bovine serum albumin
- CD40L or CD154: CD40 ligand
- CDR: Complementary determining regions
- $C_H$ : Constant domain on heavy chain
- CHO: Carbohydrate
- CIE: Counter current immunoelectrophoresis
- CRP: Complement regulatory proteins
- CsCL: Caesium chloride
- CSR: Class-switch recombination
- CTLA-4: Cytotoxic T-lymphocyte antigen-4
- CXCL12 or SDF-I : Stromal-derived factor I
- D: Factor D

- DAF: Decay accelerating factor
- DCs: Dendritic cells
- DMSO: Dimethyl Sulfoxide
- DN T cell: Double negative T cell
- DP T cell : Double positive T cell
- DP: Differential power
- EAA: Extrinsic allergic alveolitis
- EBV: Epstein Barr Virus
- ELLA: Enzyme-linked lectin assay
- Fab: Antigen binding fragment
- FACs: Fluorescence- Activated Cell Sorting
- Fc: Crystalizable fragment
- FCRs: FC receptors
- FCS: Foetal calf serum
- FcγR : IgG Fc receptors
- FITC: Fluorescein Isothiocyanate
- Foxp3: Forkhead-box transcription factor
- Fuc: Fucose
- Gal: Galactose
- GalNAc: N-acetyl galactosamine
- GATA-3: GATA binding protein-3
- GBM: Glomerular basement membrane
- GC: Germinal centre
- GITR: Glucocorticoid-induced tumor necrosis factor receptor
- GlcNAc: N-acetyl glucosamine
- H chain: Heavy chain

- HDI: Hexamethylene diisocyanate
- HFA: Hydrofluoroalkane-134a
- HLA: Human leukocyte antigen
- HP: Hypersensitivity pneumonitis
- HRCT scan: High resolution computed tomographic scan
- IC : Immune complexes
- IFN- $\gamma$ : Interferon-  $\gamma$
- Ig: Immunoglobulin
- IL: Interleukin
- IL-12R: IL-12 receptor
- ITAM : Immunoreceptor tyrosine-based activation motif
- ITC: Isothermal microcalorimetry
- ITIM: Immunoreceptor tyrosine-based inhibition motif
- JAKs: Janus-associated kinases
- L chain: Light chain
- LN2: Liquid nitrogen
- LPS: Lipopolysaccharide
- MAC: Membrane attack complex
- MASP-1: MBL-associated serine protease-1
- MASP-2: MBL-associated serine protease-2
- MASPs: Mannan-binding-protein-associated serine proteases
- MBL: mannose-binding lectin
- MDI: Methylenediphenyl diisocyanate
- MIP-1 $\alpha$  : Macrophage inflammatory protein -1 $\alpha$
- MMPs: Matrix metalloproteinases
- NF- $\kappa$ B : Nuclear factor inducing kinases

- NGF: Nerve growth factor
- NK: Natural killer
- NKT cell: Natural killer T cell
- NO: Nitric oxide
- NPV: Negative predictive value
- nTregs: Naturally occurring regulatory T cells
- OD: Optical density units
- P: Properdin
- PAS: Periodic acid schiffs
- PBMC: peripheral blood mononuclear cells
- PDF: Fresh pigeon droppings
- PDO: Old pigeon droppings
- PE: Phycoerythrin
- PFL: Pigeon fanciers' lung
- PHA: Phytohaemagglutinin
- PIM: Pigeon intestinal mucin
- PIS: Pigeon intestinal scrapings
- PMA: Phorbol 12 Myristate 13- Acetate
- PMSF: Phenyl methyl sulfonyl fluoride
- PPV: Positive predictive value
- Pre-TCR: Pre- T cell receptor
- PS: Pigeon serum
- RAG: Recombination activation gene
- RF: Rheumatoid factor
- SD: Standard deviation
- SHIP: SH2-containing inositol polyphosphate 59-phosphatase

- SHM: Somatic hypermutation
- SI: Stimulation index
- S-S: Disulfydryl bonds
- STAT-1 : Signal transducer and activator of transcription- 1
- STAT: Signal transducers and activators of transcription
- TCR: T cell receptor T cell receptor
- TD antibody: T-dependent antibody
- TDI: Toluene diisocyanate
- TGF - $\beta$ : Transforming growth factor-  $\beta$
- Th cell : T helper cell
- Th3, iTreg: Induced regulatory T cells
- TI: T-independent
- TIMP-3: Tissue inhibitors of metalloproteinases 3
- TL<sub>CO</sub>: Transferring of carbon monoxide
- TLR-4: Toll-like receptors- 4
- TM, t mucin: Trypsinised mucin
- TMA: Trimellitic anhydride
- TNF- $\alpha$ : Tumor necrosis factor- $\alpha$
- Tr1: Type 1 T regulatory cell
- TRAFs: TNF receptor-associated factors
- Treg: Regulatory T cells
- UNG: Uracil DNA glycosylase
- V<sub>H</sub>: Variable domain on heavy chain
- V<sub>L</sub>: Variable domain on light chain
- VP-ITC : One of the latest models of isothermal microcalorimetry
- $\alpha$ : Alpha

- $\alpha$ -2, 3 NeuNAc :  $\alpha$ -2, 3 N-acetyl neuraminic acid
- $\gamma$ : Gamma
- $\delta$ : Delta
- $\epsilon$ : Epsilon
- $\mu$ : Mu
- $\kappa$ : Kappa
- $\lambda$ : Lambda